

IDENTIFICATION AND MAPPING OF GENES FOR *ALTERNARIA* AND  
*PHYTOPHTHORA* DISEASE RESISTANCE IN CITRUS HYBRIDS

By

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Dedicated to the researchers and their assistants who seek the truth out there.

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By

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The inheritance of resistance to a host-specific isolate of *Alternaria alternata* f.sp. *citri* Shinn (conidia inoculation) was shown to be controlled by a single recessive allele within the citrus genome. While a backcross between *Citrus reticulata* Blanco ‘Clementine’ and LB#8-10 (a hybrid of ‘Clementine’ and ‘Minneola’) resulted in 61 resistant and 58 susceptible plants ( $X^2=0.08$ ), its reciprocal cross deviated from the expected 1: 1 ratio (87 resistant and 36 susceptible,  $X^2=21.15$ ). A cytoplasmic gene effect was suspected for the latter cross. A dominant allele, *AaM1*, of this resistance gene was found in a coupling phase linkage with two RAPD markers, P12<sub>850</sub> (15.3 cM) and AL3<sub>1250</sub> (36.7 cM) after JOINMAP computer analysis.

*Phytophthora palmivora* and *P.nicotianae* chlamydospore inoculum were used to determine tolerance to *Phytophthora* root and stem rot in citrus and trifoliolate orange hybrids. Sexual and somatic hybrids of citrus ('Ridge Pineapple', 'Gou Tou', 'Wainwright', 'Siamese sweet', 'Duncan', LB#6-1, and selection #8730) and DFG50-7 trifoliolate orange crosses varied their tolerance to *P.palmivora* and *P.nicotianae*. The results were inconsistent from one inoculation date to the next. *P.palmivora* and *P.nicotianae* showed different host range in DFG50-7 TO and Swingle. Stem rot tolerance seemed to be controlled by more than one genomic region using *in vitro* etiolated stem dual culture assay. After screening of 492 RAPD primers, three major linkage groups were discovered in Sunki x Flying Dragon (FD) and Pearl x FD hybrids combined with JOINMAP computer analysis. Map distances between RAPD markers in each linkage group were the following: First group AJ18<sub>1000</sub> and X18<sub>1000</sub> (2.3 cM); second group E16<sub>2200</sub> and AC19<sub>1300</sub> (19.5 cM), AC19<sub>1300</sub> and AM3<sub>700</sub> (12.6 cM); and third group AD10<sub>400</sub> and AB1<sub>400</sub> (14.6 cM) . The mode of inheritance of tolerance to *Phytophthora* spp. within the citrus or trifoliolate orange genome could not be determined, though the results supported polygenic control of the disease tolerance.

## CHAPTER I INTRODUCTION

Citrus is one of the most economically important fruit crop groups in the world. Northeast India, north Burma, and south China might be considered the centers of origin of citrus species (Gmitter and Hu 1990). However, the taxonomy of citrus is a controversial subject. From these regions the production of citrus has spread to all tropical and subtropical zones in the world. Producing regions now occupy a belt extending around the globe between 35°N and 35°S latitude (Burke 1967).

World total citrus production increased from 79.4 million MT in 1989 to 101.9 million MT in 1998 (Davies and Albrigo 1994, FAO 1998). Although there has been a decrease in grapefruit and lemon production, orange, mandarin, and lime production showed an increasing trend in Florida between 1994 and 1998.

Several disease agents can cause drastic decreases in fresh and processed citrus fruit production in Florida, such as citrus tristeza virus (CTV), citrus nematode (CN), blight, *Phytophthora*, and *Alternaria*. Incorporation of disease resistance in citrus cultivars is a very important objective in breeding programs. Some disease resistance is controlled by a single gene (qualitative inheritance usually by a single major dominant region in the genome), while some others appear to be controlled by multiple genes (quantitative inheritance).

Disease resistance screening methods that are reliable across species, plant age, environments, and time are required in order to have an accurate phenotypic estimation for breeding of desirable traits. Sexual hybridization is the most extensively used breeding method for most crop species. Nevertheless, evaluation of the desired traits among the progeny of perennial fruit crops takes more time because these species have a relatively longer juvenility period compared to annual plants. Natural outbreeding, self-incompatibility, and inbreeding depression can make breeding difficult and challenging for fruit crops. In addition, many of the methods of screening citrus plants are very costly and inefficient, and so their utilization in large scale breeding programs is prohibitive. An alternative approach is the utilization of marker-assisted selection (MAS), whereby resistant or susceptible plants can be distinguished on the basis of associated molecular markers linked to the genes conferring resistance. This is an indirect method of selection, based on markers and not plant phenotypes directly.

Several types of markers have been developed in citrus including leaf isozymes, RFLPs, RAPDs, SCARs, SSRs, CAPS, and STS. These markers have been used to construct total genomic maps in citrus hybrid families, which can be guides to locate single genes of interest in scion and rootstock breeding programs, e.g., resistance to CTV and citrus nematode, nucellar embryony, and dwarfing in citrus (Gmitter *et al.* 1992). These maps also allow the study of quantitative traits, such as cold hardiness and salt tolerance. The simplest of these marker systems for genome map construction are RAPDs (Welsh and McClelland 1990, Williams *et al.* 1990), and use the bulk segregant analysis

(BSA) (Michelmore *et al.* 1991). These techniques are applicable for identification of

*Alternaria* and *Phytophthora* disease resistance regions in the citrus genome.

The objectives of this research were:

1. To evaluate different citrus hybrid families for their resistance to *Alternaria* brown spot disease by detached and attached leaf assays, and to ascertain the inheritance of disease resistance;

2. To evaluate different citrus and trifoliate orange sexual and somatic hybrids for their tolerance to *Phytophthora* stem and root rot diseases, by *in vitro*, greenhouse, and growth room experiments, and to ascertain the inheritance of disease tolerance;

3. To develop RAPD markers associated with genes for resistance to *Alternaria* brown spot and *Phytophthora* stem rot.

## CHAPTER 2 REVIEW OF LITERATURE

### Alternaria Diseases in Citrus

Fungi in the genus *Alternaria*, which includes pathogens on living organisms and saprophytes on organic matter, belongs to the Fungi Imperfecti, the order Hyphomycetes, and the family Dermatiaceae (Rotem 1994). *Alternaria* brown spot disease caused by *Alternaria alternata* f.sp. *citri* Ellis and Pierce (formerly *Alternaria citri* Ellis) of *Citrus reticulata* Blanco 'Emperor' mandarin was first recorded in California in 1882 (Whiteside 1979), in coastal districts of New South Wales, Australia in 1903 (Kiely 1964), and in Florida in 1911 (Whiteside 1979). Brown spot disease was reported in a few widely separated 'Dancy' mandarin groves in Polk and Highlands counties in Florida in 1974. *Alternaria* disease was seen on 'Minneola' tangelos in the central plain of Israel (1989) (Solel 1991) and in the Çukurova region in Turkey (1995) (Canihos *et al.* 1997). This disease is more severe on 'Minneola' than 'Orlando' tangelos, which are sister cultivars from the same cross between 'Duncan' grapefruit (*Citrus paradisi* Macf.) and 'Dancy' mandarin (*C. reticulata* Blanco) (Hodgson 1967, Whiteside 1979, Motohashi *et al.* 1992). The pathogen that causes *Alternaria* brown spot on 'Dancy' and 'Minneola' foliage and fruit is a host-specific strain of *A. alternata* f.sp. *citri* (Whiteside 1979). From Australia, a strain of *A. alternata* isolated from 'Murcott' tangor had an identical host range to that of

the 'Emperor' mandarin strain (Hutton and Mayers 1988). Most of the progeny of 'Murcott' x 'Ellendale' and 'Murcott' x 'Imperial' crosses were susceptible to *A.alternata*. Another strain of this fungus is the one that causes leaf spot, defoliation, and die-back of rough lemon (*Citrus jambhiri* Lush.) and Rangpur (*C.reticulata* hybrid) seedlings (Whiteside 1993b). Therefore, there are two major isolates for brown spot disease which specifically affect either mandarins or lemons. A different strain of *A.alternata* f.sp. *citri* causes the post-harvest fruit disease known as black rot or center rot, but does not cause any injury to foliage or fruit in citrus groves (Whiteside 1986).

#### Disease Cycle, Symptomology, and Control of *Alternaria*

*Alternaria* spp. produce airborne conidia for dispersal. The pathogen survives in stem lesions, infected leaves, and in the button or styler end of the fruit. No sexually reproductive form of the fungus has yet been found. The symptoms on fruit, stem, and leaves vary from dark, large, necrotic, blighted, sunken areas to small, circular spots (Whiteside 1993a, Timmer 1999a). A toxin produced by the fungus rapidly kills a large portion of infected leaves and occasionally causes die-back of entire shoots. It produces conidia on lesions on mature leaves remaining in the canopy or leaves fallen to the grove floor. Conidia germination, infection, and symptom development may occur from 24 to 36 hours (Timmer 1999a), and spore release starts when humidity levels drop from 100 % to 50 % with a large release at 20 % humidity (Timmer *et al.* 1998).

Some fungi release toxins following inoculation and during the penetration process into host tissues. *Alternaria* spp. produce host specific toxins (HSTs) of which

two major groups exist in citrus. HSTs of *A. alternata* f.sp. *citri* were isolated from mandarin, namely *A. citri* tangerine (ACT)-toxins (Gardner *et al.* 1986, Kohmoto *et al.* 1993) and from rough lemon, namely *A. citri* rough lemon (ACR)-toxins (Gardner *et al.* 1985, Kono *et al.* 1985, Akimitsu *et al.* 1989). The target site of the ACT-toxins is on the plasma membrane (Kohmoto *et al.* 1993, Otani *et al.* 1995, Walton 1996) and that of the ACR-toxin 1 is on the mitochondrion (Kohmoto *et al.* 1993, Otani *et al.* 1995, Walton 1996, Akimitsu *et al.* 1998).

Many management practices are helpful in reducing the severity of *Alternaria* disease in susceptible cultivars. In new groves, use of disease-free trees and less vigorous rootstocks is recommended. In existing plantings, well-timed irrigation, avoidance of excessive fertilization, and frequent light hedging are the appropriate cultural practices. Copper fungicides are the only products registered for *Alternaria* disease control (Timmer 1999b).

Citrus cultivars vary in their genetically inheritance brown spot resistance. This disease has caused serious losses in fruit yield and pack-out of 'Dancy' and 'Minneola' trees in Florida, Israel, South Africa, and Turkey. On these cultivars, disease injury to twigs and defoliation has also been severe enough to cause concern. Occasionally, the same disease can be economically severe on 'Orlando'. All other commercially important citrus cultivars in Florida, except 'Murcott' and 'Sunburst', appear to be resistant to the host-specific strain (Whiteside 1979). According to a recent report from filter paper disc inoculated detached leaves (Solel and Kimchi 1997), addition to the cultivars above, 'Idith', 'Kara', 'King', 'Mikhal', 'Nova', 'Page', 'Satsuma', 'Wilking' mandarin hybrids;

'Marsh' grapefruit; 'Oroblanco' grapefruit X pummelo hybrid; 'Shamouti', 'Valencia', 'Washington' navel sweet oranges; Calamondin; and Volkamer lemon are susceptible to a brown spot disease isolate from 'Minneola.' Preliminary field observations showed that 'Clementine' mandarin is very resistant to *Alternaria* disease, and it was suggested that 'Clementine' be used in breeding for *Alternaria* resistant cultivars (Schutte 1993, L. W. Timmer per. com.). Susceptibility appears to be a dominant trait which is transferred from 'Dancy' to its progeny, and elimination of cultivars that are derived from crosses with 'Dancy' as one parent could be one of the reliable disease control measures at the breeding level (Kohmoto *et al.* 1991).

#### Disease Assay Techniques for *Alternaria* Resistance Breeding

Inoculation methods differ for determining the resistance reaction of plants to causal disease agents *in vivo* or *in vitro*, according to the plant-disease complexes. Attached (intact) or detached (cut) plant organs have been used for disease resistance screening experiments with infectious spores or toxins of organisms.

The plants need to be cut back before the expected time of *Alternaria* inoculation in order to obtain enough uniformity, young, and tender leaves. Shoot expansion stage is a very important factor when determining the resistance of different cultivars since the fungus most readily infects the young tender leaf and stem tissues. Older leaves have been shown to be less susceptible to the fungus (Gardner *et al.* 1986, Solel and Kimchi 1998). The inoculation surface is also another factor affecting fungal infection. Brown

spot lesions developed faster on the lower (abaxial) surface, where stomata exist, than upper the (adaxial) surface of the leaves.

In attached leaf assays, inoculation of potted plants under greenhouse conditions provides confidence for screening resistance. Whole plants are inoculated by spraying with conidial suspension. After 24h under high humidity condition, disease symptom appears. However, conidial inoculations on the plant leaves can cause die-back on the new shoots of susceptible cultivars because of the disease pressure (Canihos *et al.* 1998). Fungus inoculation can also be achieved by placing spores on moist sterile cotton and taping the cotton on the underside of citrus leaves *in vivo* (Gardner *et al.* 1986).

Detached leaf assays (*in vitro*), on the other hand, are easier to conduct than the attached leaf assays, and can give faster reading disease resistance of a particular plant. Drops of conidia suspensions are placed on the lower surfaces of cut citrus leaves (Kohmoto *et al.* 1991, Canihos *et al.* 1998). Nevertheless, this method requires more time to collect and label the individual leaf samples. Because the leaves are detached from their source, translocation system functions are disrupted; nonetheless, no difference was observed in disease severity between detached and intact leaves, or between leaves incubated in darkness and under natural light (Solel and Kimchi 1998). The midribs of young citrus leaves were removed, and lower surface of the leaf lamina was scratched near the center with a needle. A drop of conidial suspension was placed on each wounded site. The leaves were incubated on moistened mats in a moist chamber for 48 h at 25 °C (Kohmoto *et al.* 1993) or at 27 °C (Akimitsu *et al.* 1989). Another experiment used basal ends of young citrus shoot-tip cuttings, placed in diluted cell-free filtrates, and incubated

at 22°C under fluorescent lights to encourage transpiration. Cuttings were observed within 48 h for toxic effects (Kohmoto *et al.* 1979). An *in vitro* spray inoculation technique has been used as well. Leaves were placed inside plastic chambers (crispers) 300 x 230 x 100 mm misted inside to maintain the relative humidity near 100 %. Leaves were misted with the conidial suspension using a small hand sprayer or a chromatography sprayer. Lesions on each leaf were observed after 36 hours of inoculation. Placing conidial (Canihos *et al.* 1998) droplets using a micropipet also gave precise location of infection comparing to hand sprayers. Using host-specific toxins (HSTs) might be an alternative tool to test for resistance of commercial citrus cultivars without risking the introduction and spread of the pathogen (Kohmoto *et al.* 1991).

### *Phytophthora* Diseases in Citrus

The taxonomic classification of *Phytophthora* spp. is controversial. *Phytophthora* spp. belong the kingdom Chromista, the phylum Peronosporomycetes, the order Pythiales, the subclass Peronosporomycetidae, and the family Pythiaceae. The concept of evolution and phylogenetic relation of oomycetes to the heterokont algae has been supported by ultrastructural studies and small-subunit ribosomal RNA gene sequences, which is in agreement that the oomycetes differ from the true fungi (Erwin and Ribeiro 1996). Among these, *P. nicotianae* (syn. *P. parasitica*), *P. citrophthora*, and *P. palmivora* are responsible for the most serious soil borne diseases of citrus (Graham and Menge 1999).

Commercially important outbreaks of gum diseases started in Spain in 1832 and Portugal in 1845. During the 1850's and 1860's, foot-rot-type disease became

economically important throughout the Mediterranean area and was reported in the Americas and Southern Hemisphere (Graham and Timmer 1992). Root and foot rot diseases were responsible for the introduction of rootstocks to citriculture during the mid 1800's (Castle 1987).

In the 1920's to 1940's, foot rot became one of the most important diseases in the central-ridge area of Florida when extensive plantings were made primarily on rough lemon rootstock. This demonstrated the importance of using *Phytophthora*-tolerant rootstocks (Graham and Timmer 1992). In recent years, yield losses from fibrous root rot and foot rot were estimated to range between 3 - 6 % per year (or \$76 million per year) without fungicide treatments in the United States (Graham and Menge 1999).

#### Disease Cycle, Symptomology, and Control of *Phytophthora* spp.

Under favorable conditions of high moisture and temperature, infected roots or decaying fruit produce sporangia, which release motile zoospores. Upon contact with roots, zoospores encyst, germinate, and then infect the elongation zone. Chlamydospores, which persist in soil for long periods, germinate indirectly to produce sporangia and zoospores or directly to produce mycelia. When both mating types of *P. nicotianae* are present, oospores may be produced. *P. citrophthora* and *P. palmivora*, which produce abundant sporangia on fruit and leaf surfaces, are commonly dispersed by splashing or wind-blown rain (Graham and Menge 1999). Oospores of these two species have not yet been found in citrus soils, but can occur with *P. palmivora* mating of A<sub>1</sub> and A<sub>2</sub> types.

*Phytophthora* species are common to all citrus-growing regions causing damping-off, fibrous root rot, foot rot and gummosis, and brown rot of fruit. Until the true leaves emerge and the stem tissue lignifies, young seedlings are susceptible. The collapse of seedlings occurs when the pathogen infects and girdles the stem above the soil line.

*Phytophthora* species cause decays of fibrous roots by sloughing their cortex, leaving only the stele, and giving the root system a stringy appearance. The rot can be severe on susceptible cultivars in infested nurseries. Fibrous root rot of highly susceptible rootstocks also causes tree decline and yield losses in mature orchards. *Phytophthora* species infections can rapidly girdle and kill nursery trees and are also capable of killing older trees as well. However, large trees are usually only partially girdled, and this leads to canopy decline, defoliation, and twig die-back. Foot rot occurs in the scion bark and extends to the bud union. Gummosis occurs where the inner bark of scion is damaged. Lesions can spread around the trunk and slowly girdle the tree. Brown rot infection of fruit produces a light brown and not sunken decay below the adjacent rind. In storage, infected fruit has a characteristic pungent, rancid odor (Graham and Menge 1999).

Most commercial rootstocks, with the exception of sour orange and Swingle citrumelo, are susceptible to *P.nicotianae*. However, soil stresses including excessive water, fine-textured soils (>20 % clay content), high pH, calcareous deposits, and destructive root pests, such as citrus burrowing nematode (*Radopholus similis*) and root weevil (*Diaprepes abbreviatus*) reduce resistance of Swingle to *Phytophthora* spp. (Graham 1998). The infection and colonization of zoospores in the fibrous roots of rootstock cultivars differ (Widmer *et al.* 1998b). After 48 and 72 h, *P.palmivora* hyphae

colonized the cortex of sour orange more extensively than the cortex of trifoliate orange.

*P. palmivora* also colonized both hosts more extensively than *P. nicotianae*.

Cultural and chemical disease management techniques need to be considered according to the growth stage of groves. For establishing new groves, choosing *Phytophthora* tolerant rootstocks like sour orange or Swingle citrumelo; budding higher on the rootstock; practicing proper irrigation, drainage, and fertilization; using clean equipment; and constructing buffer zones between infested and non-infested blocks in planting sites have been recommended for prevention of foot rot (Graham and Timmer 1999). Citrus seeds can be treated at 50°C for 10 min prior to planting to avoid introducing the fungi (Graham and Menge 1999). Metalaxyl and Fosetyl-AL have been evaluated for control of *P. nicotianae* in newly planted sweet orange trees on sweet orange rootstock. Both fungicides reduced foot rot incidence, but none of the treatments increased growth of the trees (Timmer and Castle 1985). Aliette, Ridomil, or copper products can be applied to young non-bearing trees and mature bearing trees according to manufacturers' recommendations (Graham and Timmer 1999). In the late 1800s and early 1900s, there was a major shift from seedling trees and from trees on susceptible lemon, lime, and sweet orange as rootstocks, to trees budded on tolerant sour orange rootstock (Graham and Timmer 1992). However, CTV susceptibility of sour orange has led the citrus industry toward other compatible, stable, and high yielding rootstocks. Since 1960's, sexual hybrids of trifoliate orange with citrus have been under investigation as promising rootstocks for citriculture with *Phytophthora* spp. tolerance. Recently, Wutscher and Bowman (1999) reported that 'Valencia' sweet orange produced more fruit

on Vangasay lemon, HRS 812 (a hybrid of Sunki x Benecke trifoliate orange), and HRS 942 (a hybrid of Sunki x Flying Dragon) rootstocks than some other 18 rootstocks tested under *P. nicotianae* pressure.

### Disease Assay Techniques for *Phytophthora* Resistance Breeding

*Phytophthora* spp. disease resistance tests are conducted differently because of the variable nature of *Phytophthora* species' life cycle, mode of infection, and host-organ selectivity. *In vivo* disease assay methods for root and foot rot have been a challenge since the experiments began to identify tolerant rootstocks and scions. So far, true rootstock resistance has not been found against every *Phytophthora* spp. among citrus, its hybrids, and near relatives. Trifoliate orange (*Poncirus trifoliata* (L.) Raf.) selection 50-7 is resistant to *P. nicotianae*, but not to *P. palmivora*. The citrus rootstocks vary for their level of disease exclusion after invasion of the fungus. Because of this reason, the term 'tolerance' will be used in this study.

Different types of inoculation have been used to test the tolerance to root rot disease, caused by *P. nicotianae*, a common soilborne pathogen in citrus production. In early experiments, citrus seedlings were submerged either in zoospore suspension culture (Klotz *et al.* 1958a, Klotz and DeWolfe 1960, Carpenter and Furr 1962, Broadbent *et al.* 1971, Hutchison and Grimm 1972, Cameron *et al.* 1972, Grimm and Hutchison 1973, 1977, Smith *et al.* 1987); or in mycelia, sporangia, and zoospore mixtures (Furr and Carpenter 1961, Grimm and Whidden 1962) for one or two days. Then, the seedlings were planted to infested soil for the incubation period.

For evaluating the tolerance of citrus rootstocks to root rot, citrus seedlings were grown in either four- to five-inch pots (Tsao and Garber 1960), cylindrical plastic containers (Cameron *et al.* 1972), or three-liter plastic pots (Ippolito *et al.* 1996). Three to six weeks after inoculation, root systems were evaluated according to their injury with a 0-5 rating scale. In another experiment, citrus root systems were transplanted into the center of 15-cm-diameter clay pots containing Candler fine sand soil with 0.0, 0.5, 1.0, 5.0, and 10.0 propagules of *P.nicotianae* per cm<sup>3</sup> of soil. The experiment was continued for six weeks (Graham 1990, 1995a). Soil was flooded for the first three days by placing a dish under each pot and keeping the dishes full of water each day. On the third day, the water in each dish was poured out and watering during the subsequent four days occurred only when the soil surface was dry. The watering cycle was maintained for the remainder of the experiment. Propagule density of *P.nicotianae* was determined and expressed as colony-forming units (CFU) per cm<sup>3</sup> of soil or per mg root weight (Graham 1995a).

Citrus seedlings grown in separate containers were flooded from the bottom with mycelial fragment suspension (Tsao and Garber 1960), or zoospore suspension (Cameron *et al.* 1972). The citrus seedlings were removed from the sand medium and washed after two weeks. The roots were immersed in a 1% solution of 2,3,5-triphenyltetrazolium chloride for 24 h. Root damage could be expressed as a percentage of a given root mass which remained unstained (Cameron *et al.* 1972). For screening of different citrus genotypes with *P.nicotianae* chlamydospores of 10-50 propagules/cm<sup>3</sup>, 150 cm<sup>3</sup>-volume (Graham 1995b) or 75 cm<sup>3</sup>-volume (Widmer *et al.* 1998a) individual cone-tainers were used. Root systems were rated using a 1-5 scale (Graham 1995b), or positive for

incidence of infection if any colonies of *P.nicotianae* were detected from isolate 1 cm root segment (Widmer *et al.* 1998a). Planting the seedlings and budded cultivars in naturally infested soil contained in raised beds was a direct way of disease tolerance evaluation under natural growing conditions (Furr and Carpenter 1961, Klotz *et al.* 1968).

Foot rot disease resistance of citrus is tested using other screening techniques from root rot. Removing a portion of bark and placing a piece of agar culture disc which contains growing mycelia in contact with cambium (Klotz and Fawcett 1930, Klotz *et al.* 1958b, Klotz *et al.* 1968, Whiteside 1972, Tuzcu *et al.* 1984, Smith *et al.* 1987, Afek and Sztejnberg 1988, Tusa *et al.* 1988, Afek and Sztejnberg 1990, Feichtenberger *et al.* 1992, Ippolito *et al.* 1996); pouring zoospore suspension into watertight collars around the stem (Whiteside 1972, Smith *et al.* 1987); applying a slurry of mycelia, sporangia, and zoospores inside the bark (Grimm and Hutchison 1973); and using chlamydospores (Smith *et al.* 1987) were some of the methods employed for foot rot resistance screening. The length of browning around the inoculated area was measured.

In disease resistance tests *in vitro*, a dual culture systems have been used with diverse plant-pathogen combinations: sporangia suspension to potato leaves-*Phytophthora infestans* (Behnke 1980); zoospore suspension to citrus root pieces-*P.nicotianae* and *P.parasitica* (Widmer *et al.* 1998b); mycelial agar plugs to citrus etiolated seedling stem pieces-*P.nicotianae* (Bowman 1990, 1996), 'Femminello' lemon calli-*Phoma tracheiphila* (Gentile *et al.* 1993); purified toxin to *Brassica napus* detached leaves-*Alternaria brassicicola* (MacDonald and Ingram 1986); partially purified toxin to citrus-*P.tracheiphila* (Gentile *et al.* 1992); culture filtrate to citrus callus pieces-

*Phytophthora citrophthora* (Vardi *et al.* 1986), apple seedling apical stem pieces-  
*P.cactorum* (Plich and Rudnicki 1979).

Sour orange accessions were tested against gummosis fungus, *P.citrophthora* (Tusa *et al.* 1988). After seven days, the seeds at the beginning of germination were transferred into Petri dishes which contained filter paper moistened with 3 ml of sterile culture filtrate. The length of the healthy and diseased rootlets was measured after a 12-day incubation period. Results indicated that infectious spores or culture filtrates can be used as a selection tool *in vitro* (Vardi *et al.* 1986, Jones 1990).

Bowman (1990) used dual culture with 6-cm long etiolated *in vitro* grown citrus seedling stem pieces with *P.nicotianae* mycelia. The length (in mm) of discoloration (browning) on the stem above the surface of the medium was measured after seven days. He concluded that for many cultivars, the observed relative responses to *P.nicotianae* were in agreement with accepted field responses and other testing methods. However, in some other cases, the observed response to *Phytophthora* species was nearly opposite to known field resistance. Among citrus and trifoliolate orange hybrids, resistant, intermediate, and susceptible selections were found in populations using *in vitro* inoculation (Bowman 1996). While in some cases, fungal disease resistant screening methods using seedlings or *in vitro* growing stages of plants help to predict the resistance of these individuals in their later development stages (Byther and Steiner 1972, Behnke 1979, Helgeson and Deverall 1983, Tusa *et al.* 1988, Bowman 1990), in some other situations, the reverse is true (MacDonald and Ingram 1986, Vardi *et al.* 1986, Bowman 1990, Gentile *et al.* 1992, Prabhu and Rush 1997). Lack of agreement between *in vitro*

and *in vivo* results indicates that caution should be taken when applying the former technique for screening purposes.

### Fungal Disease Resistance in Plants

Several disease resistance mechanisms are operative in plants (Hooker and Saxena 1971, Jones 1990). Pre-infection defense mechanisms are preformed physical (cuticle) and chemical (phenolics) barriers. Phytoalexin production is one of the proposed post-infection defense mechanisms, but its mode of action in disease prevention has not been confirmed. Extracellular antifungal compounds (Jayasankar and Litz 1998), enhanced secretion of chitinase and glucanase enzymes (Gentile *et al.* 1993, Jayasankar and Litz 1998), high molecular weight pathogen-derived elicitors (glucans or glycoproteins), possession of a detoxification mechanism, or a lack of toxin-binding site (Jones 1990) can also be involved in defense response. After recognition of the pathogen (elicitor-receptor model), a multitude of plant-resistance-associated reactions is initiated: ion fluxes across the plasma membrane, the generation of highly reactive oxygen species, the phosphorylation or dephosphorylation of specific proteins, the activation of enzymes involved in strengthening of the cell wall, the transcriptional activation of numerous defense genes, the induction of phytoalexins, localized cell death at the infection sites, hypersensitivity (HS), and the induction of systemic acquired resistance in distal plant organs (Keen *et al.* 1993, Knogge 1996). Inactivation or inhibition of toxin permeability to the action site in resistant plants, and presence of receptors in susceptible plants, are the well accepted hypotheses to the specificity of host-specific toxins (Oku 1994).

There are at least four mechanistic classes of resistance genes confirmed by cloning and molecular characterization (Michelmore 1995). Resistance genes encode (1) components of receptor systems, (2) products that detoxify and deactivate compounds, (3) altered targets for pathogen-derived molecules, and (4) structural or constitutive biochemical barriers. There are also different structural domains of resistance genes, e.g., serine-threonine kinases, leucine-rich repeats (LRR), nucleotide binding sites (NBS), leucine zippers (LZ), toll/interleukin-1 receptor similarities, small region similarities, predicted extracellular LRR proteins, and transmembrane receptor kinases (Bent 1996). There has been evidence that different resistance genes are clustered and different functional alleles occur at some resistant gene loci (Keen 1990, Pryor 1987).

Although most disease resistance genes in plants are dominant in nature, there are also several examples of resistance genes that are recessive. While inheritance of *Alternaria* resistance is controlled by dominant (Yamamoto *et al.* 1985, Thomas *et al.* 1990, van der Biezen *et al.* 1995) or recessive (Kozaki 1976, Saito and Takeda 1984, Schutte 1993) genes, numerous research results suggest that inheritance of *Phytophthora* resistance is controlled by dominant genes (Haymes *et al.* 1997, van de Weg 1997, Walker and Bosland 1999).

### Disease Resistance and Genome Mapping in Citrus

Mapping the total genome of fruit crops will help to locate important genome regions which harbor desirable attributes. To know the genome of the wild relatives of the cultivated species will also give information of the useful gene locations to be

transferred without including undesirable traits. For this, molecular DNA markers are intensively applied in different plant species in order to find linkage between markers and corresponding gene loci. Because of the nature of evolution, most of the known plant traits are inherited as dominant genes, for example, in *Poncirus*, tolerance to cold (Weber 1999) and salinity (Moore *et al.* 1996, Tozlu 1997), and resistance to citrus tristeza virus (Gmitter *et al.* 1996, Deng *et al.* 1997, Fang *et al.* 1998, Fang and Roose 1999), and citrus nematode (Hutchison 1985, Gmitter *et al.* 1992, Ling 1996), appear to be controlled by dominant alleles. However, *Phytophthora* tolerance is probably controlled by several genes and is not a simply inherited characteristic (Hutchison 1985, Grosser and Gmitter 1990, Gmitter *et al.* 1992, Graham 1995b). *Alternaria* resistance is presumed to be controlled by a single recessive gene (Kohmoto *et al.* 1991) in citrus.

Interspecific and intergeneric backcross populations were used to characterize the citrus genome with isozymes (Durham *et al.* 1992, Jarrell *et al.* 1992, Luro *et al.* 1996), RFLPs (Durham *et al.* 1992, Jarrell *et al.* 1992, Liou *et al.* 1996, Luro *et al.* 1996), RAPDs (Cai *et al.* 1994, Luro *et al.* 1996), and SSRs (or microsatellites) (Luro *et al.* 1992, 1996, Fang and Roose 1996b, Kijas *et al.* 1997). During these genome mapping studies, markers have been constructed in different number of linkage groups, e.g. 9 (Cai *et al.* 1994, Weber 1999), 10 (Jarrell *et al.* 1992), 11 (Durham *et al.* 1992), and 12 (Luro *et al.* 1996).

Localized linkage maps were constructed for the citrus tristeza virus resistance gene, *Crv*, from *P. trifoliata* with RFLP (Mestre *et al.* 1997a, Fang *et al.* 1998), RAPD (Fang and Roose 1996a, Gmitter *et al.* 1996, Mestre *et al.* 1997a, Fang *et al.* 1998), and

marker systems. A novel gene, *Ctv2*, conferring CTV resistance in pummelo, *Citrus maxima* Burm. Merrill, was found (Fang and Roose 1999). Ling (1996) found six RAPD markers linked to a citrus nematode resistance gene region in a *Citrus - Poncirus* backcross population.

A single dominant gene was found from open pollinated seeds of *Poncirus trifoliata* cv. Flying Dragon, conferring dwarfing effect on grafted scions, using RAPD markers (Roose *et al.* 1994, Cheng and Roose 1995). Fang *et al.* (1997a) reported that the acidless trait in pummelo is controlled by a single recessive gene called *acitric*.

Durham (1990) found that at least seven quantitative trait loci (QTL) were responsible for freezing tolerance in a backcross population between *Citrus* and *Poncirus* using isozymes and RFLP markers. In similar studies, Tozlu (1997) associated 135 QTL for salinity tolerance. Weber (1999) located one major QTL or group of QTLs in *Poncirus* for cold tolerance using sequence-based PCR markers.

### BSA, RAPD, PCR, and Linkage Analysis

Bulked segregant analysis (BSA), random amplified polymorphic DNA (RAPD) markers, and polymerase chain reaction (PCR) technologies are common approaches to search the total plant genome to gain more information about approximate locations of disease resistance genes. In BSA, plants are separated in two groups according to their disease reaction extreme. Each bulk contains individuals that are identical for a particular trait but random at other unlinked regions. This approach can have widespread application both in selfing and in outbreeding species (Michelmore *et al.* 1991). BSA has

been used in the following plant-pathogen complexes: bean-*Uromyces appendiculatus* (Haley *et al.* 1993), bean-bean common mosaic virus (Haley *et al.* 1994b), lettuce-*Bremia lactucae* (Michelmore *et al.* 1991, Kesseli *et al.* 1993, Maisonneuve *et al.* 1994), strawberry-*Phytophthora fragariae* (Haymes *et al.* 1997), citrus nematode-*Tylenchulus semipenetrans* (Ling *et al.* 1994, Ling 1996), *Poncirus trifoliata*-CTV (Gmitter *et al.* 1996, Deng *et al.* 1997, Mestre *et al.* 1997a, 1997b, Fang *et al.* 1998), and apple-scab (Hemmat *et al.* 1998).

RAPD markers are DNA based, inexpensive, simple to use, easy to automate, less time consuming, and usually dominant markers (Welsh and McClelland 1990, Williams *et al.* 1990). They do not require radioisotope labeling, and prior knowledge of DNA sequence is unnecessary. However, non-reproducibility is the most important obstacle which is caused by imprecise matches between the oligonucleotide primers and the template DNA at the low annealing temperature. Different thermal-stable DNA polymerases and thermal cyclers can cause variability in results (Fowler *et al.* 1994, Kelly 1995, Mohan *et al.* 1997). Since they have been developed, RAPD markers have been used in different research areas such as genome mapping in *Citrus* x *Poncirus* intergeneric crosses (Cai *et al.* 1994, Luro *et al.* 1996, Kijas *et al.* 1997, Fang and Roose 1999, Weber 1999). An amplified DNA can be cloned and used as a starting point for reaching genes (McPherson *et al.* 1992).

PCR is a routinely used to amplify specific DNA sequences in molecular biology. It is a useful tool in fingerprinting in many fruit crops (Luro *et al.* 1992, Deng *et al.* 1995, Deng *et al.* 1996a, Machado *et al.* 1996, Woeste *et al.* 1996, Fang *et al.* 1997b, Autio *et*

*al.* 1998); diagnosis for genetic diseases; mutational analysis; forensic; archeology, paleontology, and evolution; mapping and sequencing of genomes (Gmitter *et al.* 1996, Fang *et al.* 1998, Weber 1999); and population genetics areas (McPherson *et al.* 1992, Howe 1995). PCR can also be used in plant-pathogen interactions for unraveling gene discovery, cloning, and genome analysis. Genomic DNA, oligonucleotide primers, deoxynucleotide triphosphate (dNTP), DNA polymerase enzyme, and reaction buffer with  $Mg^{++}$  are required components of a simple PCR (Mullis 1990, Howe 1995). To amplify unknown sequences, PCR conditions can be altered to promote multiple priming by using short primers, high  $MgCl_2$ , and low annealing temperature (Thomas 1996).

Markers from the segregating progeny of different crosses can be tested with available computer generated programs for linkage analysis, for example, MAPMAKER/EXP v3.0 (Lander *et al.* 1987, Lincoln *et al.* 1993a), MAPMAKER/QTL v1.1 (Lincoln *et al.* 1993b), and JOINMAP v2.0 (Stam and Ooijen 1995). Data files are arranged for segregating  $BC_1$  or  $F_2$  format. The minimum likelihood distance and corresponding the logarithm of odds (LOD) score among markers are taken. If the LOD score was greater than a minimum LOD threshold of 1.0-3.0 and the distance is less than a maximum recombination frequency of 25-50 cM, then the markers are considered to be linked. Recombination frequencies are corrected based on Haldane's or Kosambi's map distance function. JOINMAP gives shorter map distances than MAPMAKER, and allows to analyze data from different crosses.

### Concluding Remarks

Choosing appropriate parents for sexual crosses, observing adequate segregation among seedlings for their reaction to treatments, and applying dependable screening procedures play important roles in the beginning of any disease resistance breeding research. An integrated approach, merging classical phenotypic selection with a genetic marker-based analysis, may aid in extracting valuable genes from heterogenous populations (Paterson *et al.* 1991). The combination of plant breeding programs with sufficient number of closely linked, segregating, highly reproducible, economical, and easily used markers enhances fruit crop germplasm selection for desirable traits. Using dominant RAPD molecular markers, time consuming efforts can be shortened by marker-assisted selection (MAS) conducting many rounds of selection without the existence of pathogens (Kelly 1995, Mohan *et al.* 1997, Ribaut and Hoisington 1998) for disease resistance breeding. However, MAS can be less useful when many genomic regions need to be managed. Breeders ought to emphasize use of markers which are linked with the disease susceptibility allele in order to be as efficient selection as possible in early segregating generations (Haley *et al.* 1994a, Kelly 1995). Marker orientation with the resistance allele and the type of population are the two major determinants of RAPD markers for MAS. Coupling-phase markers (in which the situation describes the linkage between markers and genetic loci on the same chromosome) are quite useful in MAS to move traits quickly from wild relatives into commercial cultivars (Johnson *et al.* 1995). Selection against repulsion-phase (the situation is that markers and genetic loci are located in different homologous chromosomes) RAPD markers provided a greater

proportion of homozygous resistance genotypes in common beans (Haley *et al.* 1994b, Johnson *et al.* 1995, Kelly 1995) and accelerated the restoration of the domestic parent's attributes (Paterson *et al.* 1991). Nevertheless, a marker developed for a gene in one cross may not be useful in other crosses even though the same gene may be segregating in other crosses, unless the marker is very tightly linked to, or is from the gene itself. The success will depend on identifying marker(s) as close to the gene as possible for its utility across all populations. With MAS, resistance (R) genes from diverse sources can be incorporated in a single genotype for durable resistance (Mohan *et al.* 1997). Backcross breeding method can be used to fix the good characteristics of the current cultivars and incorporate the necessary disease resistance. However, improvement with backcross breeding for recessive disease resistance genes might be slow and difficult (Jones 1990). With the help of MAS, fruit crop improvement can be also achieved for disease resistance to a number of pathogens at the same time, more efficiently than by conventional systems (Grattapaglia *et al.* 1992, Kelly 1995, Staub *et al.* 1996, Gresshoff 1999). Although this technology cannot be used with thousands of individuals for commercial nursery production yet because of its high cost, it is very suitable for research and breeding purposes. In this study, the purpose will be to determine the mode of inheritance of *Alternaria* and *Phytophthora* disease resistance, to identify RAPD markers, which might be used for *Alternaria* and *Phytophthora* resistance genes in citrus, and to discuss the eligibility of these markers for MAS.

### CHAPTER 3

## PHENOTYPIC DETERMINATION OF *ALTERNARIA* DISEASE RESISTANCE IN CITRUS HYBRIDS

### Introduction

'Minneola' and 'Orlando' tangelos (*C.paradisi* Macf.'Duncan' x *C.reticulata* Blanco. 'Dancy') occupied an important portion of Florida fresh citrus production with 4.0 million boxes in 1996-1997 and 2.9 million boxes in 1997-1998 harvesting seasons. Production of 'Murcott' (Honey tangerine), another important fresh market cultivar, increased from 1.8 million boxes in 1996-1997 to 2.0 million boxes in 1997-1998. The number of tangelo and mandarin trees planted annually in the last decade fluctuated (Florida Commercial Citrus Inventory 1998). The total acreage of 'Orlando', the main cultivar, decreased 9 %, from 3773 ha in 1996 to 3432 ha in 1998. There was a 3.3 % decrease in total 'Minneola' acreage, from 1318 ha in 1996 to 1275 ha in 1998 (Annual Report 1998). Although there is not a remarkable change in the number of trees or acreage, one of the reasons for the reduction of tangelo production has been foliar fungal disease problems in Florida due to the hot and humid climate. Since 'Orlando' tangelos have been used for cross-pollination purposes for other citrus cultivars (per. com. with S. H. Futch), and because it is one of the most freeze-hardy of all commercially important citrus cultivars with the exception of satsumas (Davies and Albrigo 1994).

In recent years, citrus growers with 'Minneola' tangelos (which are very susceptible to brown spot disease) in their groves have expressed their concern about *Alternaria* sp. disease (per. com. with L. W. Timmer). *Alternaria* sp. disease causes yield reduction and young foliage losses of susceptible cultivars, especially cultivars derived from 'Dancy' mandarin, which is highly susceptible to this disease. Because of its *Alternaria* sp. resistance, *C. reticulata* Blanco 'Clementine' was chosen as one of the seed parents for this study. Its monoembryonic (one true hybrid embryo per seed) characteristic has made this cultivar suitable for extensive use for different crosses as a seed parent in breeding programs. The utilization of new genetic sources among mandarin hybrids for their brown spot resistance, while maintaining the marketable characteristics of these cultivars, would benefit Florida citrus growers.

The objective of this study was to evaluate different mandarin-type hybrid families to gain more information about the possible mode of inheritance of resistance to *Alternaria* sp., using detached and attached leaf assays.

### Materials and Methods

#### Plant Material

From July to September 1995, some of the mandarin-type hybrid families available from the Citrus Research and Education Center (CREC), Lake Alfred, FL of the University of Florida citrus breeding programs were evaluated for their resistance to *Alternaria* sp. The hybrids from the following crosses were tested for their *A. alternata*

f.sp. *citri* Shinn isolate resistance: 'Clementine' x 'Minneola', 'Clementine' x 'Murcott', 'Nakon' pummelo x 'Minneola', 'Nakon' x 'Page', 'Nakon' x LB#8-9 ('Clementine' x 'Minneola'), 'Thong Dee' pummelo x 'Minneola', 'Lee' x 'Nova', 'Lee' x 'Fairchild', 'Robinson' x 'Fairchild', 'Fortune' x 'Orlando', LB#8-4 x 'Orlando', LB#8-9 x 'Orlando', LB#8-8 x US119, LB#8-8 x USDA 15-60, LB#8-4 x 'Ortanique', LB#8-9 selfed, and LB#3-1 x LB#8-15. Ten-year-old 'Dancy' trees obtained from tissue culture somaclonal selection which were predicted to be resistant to ACT toxin (per. com. with J. L. Chandler) (kindly provided by J. W. Grosser) were included in *Alternaria* sp. resistance tests. According to information obtained from the 1995 tests, new crosses were made for evaluating *Alternaria* sp. resistance in citrus hybrids in 1996 (Table 3.1). For these, a seedy clonal selection of 'Clementine' (located in the Citrus Arboretum of the Florida Department of Agriculture and Consumer Services (FDACS), Division of Plant Industry (DPI), Winter Haven, FL) and LB#8-10 (maintained at the CREC) were used as parents in reciprocal crosses (#9660 and #9664) to obtain the hybrids for detailed investigation. LB#8-10 was obtained from a cross between 'Clementine' and 'Minneola' made by A. P. Pieringer in mid 1970s. It produces only one true hybrid embryo per seed seeds (M. K. Wendell and F. G. Gmitter, Jr. per. com.). Other crosses were made, as well, including 'Lee' hybrid x 'Nova' tangelo ('Clementine' x 'Orlando', for each parent, thus creating F<sub>2</sub> hybrids, #9662), LB#8-10 x 'Minneola' (a backcross, #9665), and LB#8-10 selfed (also for an F<sub>2</sub> family, #9666). From the crosses #9660, #9662, #9664, #9665, and #9666, 913, 345, 727, 758, and 28 seeds were obtained, respectively.

Seeds were extracted and sown in Multipot #3, 96-cavity, white plastic seed trays 609 x 355 x 121 mm (United Agri Products, Waverly, FL) with Fafard® soil mix 4-P (Conrad Fafard®, Inc., Agawam, MA). The hybrids from the cross #9660 were planted on September 6, 1996 (170 seeds) and October 16, 1996 (142 seeds); unplanted seeds were stored at 4°C. From six randomly chosen fruits, 119 seedlings were tested with detached and attached leaf assays using the Shinn isolate of *A. alternata* f.sp. *citri*. Leaf samples from these hybrids were used in the RAPD-PCR analysis for identifying brown rot disease associated DNA markers (Chapter 5). The seeds from the cross #9664 were planted on September 13, 1996 (95 seeds) and October 2-3, 1996 (632 seeds). After germination, 123 random hybrids were tested with the Shinn isolate for their resistance to brown spot disease. The seeds of the crosses #9662, #9665, and #9666 were sown in September and October 1996, and 170, 183, and 11 of the seedlings, respectively, were inoculated with conidial suspension of *A. alternata* f.sp. *citri* Shinn isolate. Regular cultural practices of irrigation, fertilization, pest and disease control were given to the seedlings during their growing period in the greenhouse.

#### Conidia Production and Plant Inoculations

Production of inoculum and inoculation techniques were modified from Whiteside (1976) and Canihos *et al.* (1998). The Shinn isolate of *Alternaria alternata* f.sp. *citri* was obtained from a leaf lesion in a Minneola tangelo grove near Polk City, FL on 25 April 1995 by L. W. Timmer. *Alternaria alternata* f.sp. *citri* RL-4 was isolated from a rough lemon leaf from a grove near Haines City, FL by L. W. Timmer in July 1992. To assure

that experiments were conducted with a cultivar-specific isolate, the RL-4 isolate was included in these preliminary experiments. Monoconidial cultures were obtained and maintained either on sterilized Whatman filter papers (Whatman Int. Ltd., Springfield Mill, Maidstone, Kent, UK) or on silica gel desiccant (Fisher Scientific, Fair Lawn, NJ) at 4°C. Cultures were grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) for 3 to 5 days at 27°C. The surface of the plates was scraped with a scalpel to remove aerial mycelia and the colony was cut into approximately 2-mm square pieces. Small pieces were transferred to each Petri plate of a sporulation medium consisting of 30 g CaCO<sub>3</sub>, 20 g sucrose, and 20 g agar per liter of distilled water (Canihos *et al.* 1998). Two ml of sterile, distilled water was added to each plate. The plates were sealed with Parafilm®, and incubated for 3 to 5 days at 27°C. Conidia were collected by adding approximately 10 ml of sterile, distilled water to each plate and gently scraping the surface of the colonies with a small paint brush and then filtering the suspension through two layers of cheesecloth to remove mycelial fragments. Conidia were washed two times in sterile, distilled water into 50 ml Nalgene Oak Ridge Polycarbonate Centrifuge Tubes with round bottoms (Nalge Nunc International, Rochester, NY) by centrifugation at 6000 rpm for 10 min to remove additional mycelia and any toxin carried over from the culture plates. The pellet in the bottom of the tubes was re-suspended in 40 ml of double distilled water and gently vibrated for few seconds. Conidia suspension was adjusted to 10<sup>4</sup> conidia/ml using a hemacytometer. Glycol was added to the tubes (20 % of the total volume). The tubes were stored at 4°C until used.

### Detached and Attached Leaf Assays

Young leaves from hybrids and propagated parent plants grown in the greenhouse were used for conidia inoculation experiments. When the seedlings produced new leaflets, the single, tender, leaf (1-2 cm long) closest to the shoot tip was collected from each plant and brought to the laboratory. Leaves were placed on their adaxial sides on metal screens within 300 x 230 x 100 mm rectangular containers with tight fitting lids (crispers) (T295C, Tri-State Molded Plastic, Inc., Dixon, KY). Leaves were misted with the conidia suspension on their abaxial sides using a small hand sprayer or a chromatography sprayer (Crown Spra-Tool, North America Professional Products, Woodstock, IL). In some instances, droplets of conidial suspension were placed on the abaxial surface of the leaves by using a pipetter (Rainin Instrument Co. Inc., Woburn, MA). Double distilled water was sprayed or dropped onto control leaves. Distilled water was added to the containers to maintain humidity. Lesions on each leaf were observed three days after inoculation. The parent plants were included at each time of the inoculation. Plants were scored susceptible if any symptoms were observed. Leaves from symptomless plants were inoculated a second time to verify their resistance to the disease.

For the attached leaf assay, hybrids which were grown and maintained in the 96-cavity-white plastic trays were cut-back, leaving 2-3 nodes above the soil level in order to synchronize new flush. The seedlings were brought into a growth chamber (Percival MFG. Co., Boone, IO) and kept at 27°C, 100 % RH, in the dark. Conidia suspensions were sprayed as described above. Three days after inoculation, plantlets were scored susceptible or resistant depending on the presence or absence of symptoms.

### Results

In 1995, some of the available citrus hybrid plants (Table 3.1) were tested for their resistance to have a better understanding of the possible mode of inheritance of brown spot resistance. All of the 15 selected 'Dancy' somaclones, which were selected from *Alternaria* sp. culture filtrate assay *in vitro*, were susceptible to Shinn isolate. There was no symptom observed on the ddH<sub>2</sub>O treated control leaves. All of the hybrids obtained from 'Minneola' crosses with 'Clementine', and with 'Nakon' and 'Thong Dee', showed a positive reaction to the disease when they were inoculated with the Shinn isolate. Hybrids between 'Lee' and 'Nova' gave a predicted F<sub>2</sub> type of Mendelian segregation ratio of 1 resistant to 3 susceptible progeny. The same segregation ratio was true for the 'Lee' x 'Fairchild' hybrids. Most of the 'Orlando' hybrids and the hybrids from 'Robinson' and 'Fairchild' cross were susceptible. Disease symptoms in the 'Orlando' crosses developed late, and lesions were fainter color than the 'Minneola' crosses. While 'Minneola' was susceptible, 'Clementine' did not show any symptoms at any time. Thus susceptibility appeared dominant homozygous in 'Minneola' and resistance recessive homozygous in 'Clementine', against the Shinn isolate. In all cases, the Shinn isolate infected only 'Minneola' leaves whereas the RL-4 isolate infected only rough lemon (data not presented). Therefore, the Shinn isolate was cultivar-specific.

In 1996, detailed tests were conducted with reciprocal crosses of 'Clementine' with LB#8-10 ( #9660 and #9664), as well as LB#8-10 with 'Minneola', 'Lee' with 'Nova', and LB#8-10 selfed (Table 3.2). The number of resistant plants was 61 which showed no symptoms and 58 susceptible plants showed symptoms ( $\chi^2=0.08$ ,  $P \leq 0.05$ )

Table 3.1. Tests for *A. alternata* f.sp. *citri* Shinn resistance of citrus hybrids in 1995.

Crosses	Model	R	S	$\chi^2$		
				0:1	1:1	1:3
'Clementine' x 'Minneola' (LB#8- ) <sup>y</sup>	ss x SS	0	9	0.00	-	-
'Fortune' x 'Orlando'	Ss x Ss	0	12	0.00	-	-
'Nakon' x 'Minneola'	ss x SS	0	34	0.00	-	-
'Thong Dee' x 'Minneola'	ss x SS	0	32	0.00	-	-
'Nakon' x LB#8-9	ss x Ss	11	16	-	0.93	3.57
LB#8-8 x US119	Ss x ss	9	10	-	0.05	5.07*
'Clementine' x 'Murcott' (LB#3- ) <sup>x</sup>	ss x Ss	1	3	-	-	0.00
'Lee' x 'Nova'	Ss x Ss	9	28	-	9.76*	0.01
'Lee' x 'Fairchild'	Ss x Ss	4	18	-	8.91*	0.55
LB#3-1 x LB#8-15	Ss x Ss	4	19	-	9.78*	0.71
LB#8-9 selfed	Ss x Ss	3	6	-	1.00	0.33
LB#8-4 x 'Ortanique'	Ss x ss	13	20	-	1.48	3.65*
LB#8-8 x USDA15-60	Ss x ss	6	4	-	0.40	1.20
'Robinson' x 'Fairchild'	Ss x Ss	2	23 <sup>w</sup>	-	17.64*	3.85*
'Nakon' x 'Page'	ss x Ss	50	22 <sup>w</sup>	-	10.89*	1.19
LB#8-4 x 'Orlando'	Ss x Ss	1	13 <sup>w</sup>	-	10.29*	2.38
LB#8-9 x 'Orlando'	Ss x Ss	5	59 <sup>w</sup>	-	45.56*	10.08*

<sup>z</sup> Segregation ratios between resistant (R) and susceptible (S) plants.<sup>y</sup> All LB#8- selections were from 'Clementine' x 'Minneola' cross.<sup>x</sup> All LB#3- selections were from 'Clementine' x 'Murcott' cross.<sup>w</sup> Aberrant crosses.\* Significant at  $P \leq 0.05$ .Table 3.2. Tests for *A. alternata* f.sp. *citri* Shinn resistance of citrus hybrids in 1996.

Crosses	Model	R	S	$\chi^2$		
				0:1	1:1	1:3
9660 DPI6-7 'Clementine' x B7R2T7 LB8-10	ss x SS	61	58	-	0.08	-
9662 DPI5-10 'Lee' x B11R6T2 'Nova'	Ss x Ss	42	128	-	43.51*	0.01
9664 B7R2T7 LB#8-10 x DPI6-7 'Clementine'	Ss x ss	87	36	-	21.15*	1.20
9665 B7R2T8 LB#8-10 x DPI4-10 'Minneola'	Ss x SS	0	183	0.00	-	-
9666 B7R2T6-7 LB#8-10 selfed	Ss x Ss	5	6	-	0.09	-

\* Significant at  $P \leq 0.05$ .

with in agreement with the expected 1:1 ratio in 'Clementine' x LB#8-10 backcross. The segregation of disease resistance (87 resistance: 36 susceptible) within the LB#8-10 with 'Clementine' backcross population was different from the reciprocal cross. The Chi-square value,  $X^2=21.15$ , for 1:1 segregation was highly significant at the  $P \leq 0.05$  level. The 'Lee' x 'Nova' cross revealed 42 resistant and 128 susceptible progeny ( $X^2=0.01$  for 1:1 ratio). All 183 hybrids from the LB#8-10 x 'Minneola' cross were susceptible. Selfing LB#8-10 did not yield enough sexual hybrids for disease resistance evaluation (5 resistant and 6 susceptible).

### Discussion

According to the results from testing segregating families in 1995 and 1996, one recessive gene for resistance to brown spot disease was proposed, based on the heritage of hybrids from 'Clementine' (ss), and the  $F_1$  and  $F_2$  type Mendelian segregation ratios. The Chi-square test with the 'Clementine' (ss) x LB#8-10 (Ss) backcross population supported the hypothesis that resistance to the Shinn isolate was controlled by a single recessive allele inherited from 'Clementine' mandarin. However, the reciprocal backcross resulted in a distorted segregation ratio. One possible explanation for these results could be that different cytoplasmic genes play a role, depending on the direction of the cross. Paternal mitochondrial effect has been speculated (Moreira *et al.* 1998). However, distorted segregation ratios are common in citrus (Durham *et al.* 1992, Cai *et al.* 1994). The distorted backcross segregation ratios do not contradict strongly the evidence from several other crosses supporting the hypothesis that *Alternaria* resistance is controlled by

a single recessive gene. Recessive allele controlled plant disease resistance is rare among plants, but it has been documented (Ebba and Person 1975, Kozaki 1976, Miklas *et al.* 1996, Saito and Takeda 1984, Schutte 1993, Haley *et al.* 1994b). Regardless of the seed parent, all progeny derived from 'Minneola' (SS) as pollen parent were susceptible. Crosses using 'Orlando' as pollen parent were also susceptible, so 'Orlando' is SS. The few resistant progeny from 'Orlando' crosses may be from open pollination, because flowers were not protected and cannot exclude chance open pollination causing aberrant segregation ratios. 'Dancy' (SS) somaclones were susceptible in the field, although they were selected from *Alternaria* challenged *in vitro* toxin cultures.

In the *Alternaria* resistance tests performed, it was found that there were identical resistance ratings regardless of inoculation methods used (attached vs. detached; hand-sprayer vs. application of droplets by pipetter). These results were in agreement with previous studies for evaluation of cultivar susceptibility (Pegg 1966, Kohmoto *et al.* 1979, Solel and Kimchi 1997). The conidia suspension spray method under sufficient humidity environment at the earliest possible growing stage of the seedling progeny will speed the selection efficiency discarding susceptible individuals and providing smaller greenhouse space for evaluation. The resistance conditions of mandarin cultivars need to be tested with different *A. alternata* isolates (Solel and Kimchi 1997). Then, sexual hybridizations should be made for transferring *Alternaria* sp. resistance gene(s) from the resistant cultivars to new commercial cultivars (Kohmoto *et al.* 1991, Schutte 1993).

The 'Clementine' x LB#8-10 backcross hybrids were chosen for identifying the *Alternaria* resistance gene associated RAPD markers.

## CHAPTER 4

### PHENOTYPIC DETERMINATION OF *PHYTOPHTHORA* ROOT AND STEM ROT DISEASE TOLERANCE IN CITRUS HYBRIDS

#### Introduction

During 1997-1998, 32 different rootstocks were produced in Florida citrus nurseries, but six of these accounted for 97 % of registered nursery propagation. Swingle citrumelo continues to be the most popular rootstock (more than 50 % of the nursery trees). Carrizo citrange is the second most favored rootstock (21.6 %), followed by Smooth Flat Seville (9.9 %), Cleopatra and Sun Chu Sha mandarins (8.1 %), and lemon types (7.4 %). These top six rootstocks remained unchanged in ranking from the previous year (Annual Report 1998). The popularity of Swingle citrumelo is based on its resistance to CTV-induced decline, possible tolerance to citrus blight, and strong resistant to citrus nematode and foot rot caused by *Phytophthora nicotianae*.

*Phytophthora* spp., primarily *P.nicotianae* and *P.citrophthora*, are responsible for the most important soil borne diseases for citrus production (Timmer and Menge 1993). *P.palmivora* has recently become a concern for citrus plants on Swingle citrumelo rootstock in the east coast citrus growing region (flat-woods) of Florida (Graham 1998). Like CTV, *Phytophthora*-induced diseases, are considered to be rootstock problems, even though infection of the scion at or above the bud-union can occur, resulting in decreased productivity or tree loss (Swingle and Reece 1967).

Differences in tolerance to *Phytophthora* species have been reported within *Citrus* species, as well as among related genera (Grosser and Gmitter 1990, Hutchison and Grimm 1973, Swingle and Reece 1967). Rootstocks with good *Phytophthora* tolerance include trifoliate orange, sour orange, and Swingle citrumelo (Graham 1990, 1995b, Graham and Timmer 1992, Hutchison 1974, Graham and Menge 1999). Trifoliate orange has been the primary source of genes for tolerance to *Phytophthora* species in intergeneric citrus hybrids that have found wide acceptance as rootstocks in many parts of the world (Barrett 1985, Gmitter *et al.* 1992).

The goal of this study was to determine the mode of inheritance of resistance to root and stem rot in populations of individuals from crosses of trifoliate orange (highly resistant to *P. nicotianae* and tolerant to *P. palmivora*) with citrus (susceptible to both *Phytophthora* spp. in varying degrees).

## Materials and Methods

### Root Rot Tolerance Experiment

Crosses were made in 1995 and 1996 (Table 4.1) at CREC, Lake Alfred, FL and DPI, Winter Haven, FL. The common paternal parent for these crosses was DPI9-6 TO. This plant has a sister apomictic seedling introduced from China (pers. comm. with F. G. Gmitter, Jr.) located at the Dundee Foundation Grove (DFG50-7) of FDACS. They are identical morphologically and indistinguishable by RAPD-PCR fingerprints (data not shown). This parent selection will be referred to as DFG50-7 TO. Carrizo citrange, Cleopatra mandarin, and DFG50-7 TO were used as control plants for tolerance testing.

Table 4.1. Crosses for *Phytophthora* spp. root rot disease tolerance in citrus and trifoliate orange (DFG50-7 TO) hybrids in 1995 and 1996.

Crosses	♀ Parents	No. of hybrids
9518	'Ridge Pineapple' sweet orange	19
9519-21	'Gou Tou' sour orange hybrid	15
9522	'Wainwright' pummelo	23
9523	'Siamese' sweet pummelo	11
9524	'Duncan' grapefruit	15
9648	LB#6-1 <sup>z</sup>	50
9669	8730 <sup>y</sup>	29

<sup>z</sup> LB#6-1: Hybrid of 'Clementine' x 'Hamlin'.

<sup>y</sup> 8730 : Hybrid of LB#8-9 ('Clementine' x 'Minneola') x 'Orlando'.

Seeds from the crosses were sown in Multipot #3 96-cavity white plastic seed trays 609 x 355 x 121 mm (United Agri Products, Waverly, FL) in October 1995 and October-November 1996. Soil mix #4-P (Conrad Fafard<sup>R</sup>, Inc. Agawam, MA) was used. Seedlings were fertilized bi-weekly with Peter's 20-20-20 (N-P-K) Peat-lite Special (W.R.Grace, Fogelsville, PA). True hybrids having trifoliolate leaves were transplanted to 3362-cm<sup>3</sup> black plastic bags (105 x 105 x 305 mm) (Nina Plastics, Orlando, FL) and replaced on screenhouse benches.

When the seedlings were reached 50 cm, 2-3-node semi-soft cuttings were made from each hybrid. The bottom portion of the cuttings were dipped in 3000 ppm powder indole 3-butyric acid (Hormodin 2, MSD-AGVET, Division of Merck & Co., Inc., Rahway, NJ) for a few seconds and then placed them in 38-cavity-black plastic 150-cm<sup>3</sup> tree trays (Tray Masters, Sydney, FL) containing horticultural perlite (Aero-Soil, Chemrock Corporation, subsidiary of Grefco, Inc., Jacksonville, FL) for rooting. The cuttings were kept with misting (5 sec/min) on the bench until they were rooted. At least three replicate plants were used from each individual hybrid seedling for each experiment.

Rooted cuttings of somatic hybrids from 8 citrus+citrus and 7 citrus+trifoliolate orange combinations were kindly provided by J. W. Grosser at UF-IFAS, CREC, Lake Alfred, FL. These somatic hybrids were tested for *Phytophthora* resistance, along with the sexual hybrids. From each somatic hybrid, at least 3 replications were tested. In addition, 12 and 11 different nucellar hybrids of *Citrus sunki* Hort. ex Tan. Sunki x *Poncirus trifoliata* (L.) Raf. Flying Dragon (FD) and *C.xtangelo* Pearl ('Imperial'

grapefruit x 'Willowleaf' mandarin) X FD crosses, respectively, with 10 nucellar seedling replicates of each (provided by K. D. Bowman at USDA-ARS, Whitmore Foundation Farm, Leesburg, FL), were inoculated with Pp101 isolate in the greenhouse. Swingle nucellar seedlings were used as control checks.

#### *Phytophthora* spp. chlamydospore production

The following isolates were used for chlamydospore production: *P. nicotianae* Blanton was isolated from soil, Blanton, Lake County, FL in summer 1987 by L.W.Timmer; *P. nicotianae* Wood was isolated from plant bark tissue from Felda Groves in Florida in October 1991 by L.W.Timmer; *P. nicotianae* Pn117 was isolated from soil, Overlook Groves, Ona block #2 tree #61 in Florida in July 1997 by J.H.Graham. For the plant inoculations, a mixture of Blanton, Wood, and Pn117 was used during experiments and referred to as Pn117. *P. palmivora* P99-59-1 (referred to as Pp101), was isolated from soil in Ben Hill Griffin Bass Grove in Florida in September 1996 by J.H.Graham.

The procedure for chlamydospore production, harvest, and soil infestation was modified from Mitchell and Kannwischer-Mitchell (1992), Graham (1995a), Graham and Menge (1999), and Widmer *et al.* (1998a). The chlamydospore suspension was mixed into 10 L of steam-sterilized moistened fine sand soil in a bucket which was covered with a plastic bag. Propagule levels were determined by plating 1-cm<sup>3</sup> soil samples (five plates per sample) on a selective medium containing pimarin- ampicillin- rifampicin- pentachloronitrobenzene- hymexazol (PARPH).

### Plant inoculation with Pp101 and Pn117

The root systems of the cuttings were rinsed free of potting medium with water. Soil that was steam-sterilized for at least 6 h was mixed with culture-produced chlamydospores to a density of 10-50 propagules per cubic centimeter of Candler fine sand soil (Typic quartzipsamments, pH=6.8, and 1.0 % organic matter). The root system was pruned to 5-cm in length and transplanted in 25 cm<sup>3</sup> of infested soil in 150 cm<sup>3</sup> cone-tainers (Stuewe & Sons, Inc., Corvallis, OR). Inoculated plantlets were irrigated every other day. Greenhouse and growth room ambient temperature ranged from 20 to 35°C and relative humidity ranged from 60-100 % during the experiments.

There were 16 different inoculation dates in the experiment (Table 4.2). The root systems of the plantlets were evaluated 8-12 weeks after inoculation. Disease severity was rated on a 1 to 5 scale modified from Grimm and Hutchison (1973) and Graham (1995b) (Table 4.3). Only the experiment dates that showed separable disease scoring values among the hybrids were analyzed by ANOVA using the PROC GLM procedure of Statistical Analysis System (SAS Institute, Inc., Cary, NC). Duncan's Multiple Range test ( $P \leq 0.05$ ) was used for cross means separation according to each disease scoring date. Orthogonal contrasts were calculated between cross means and controls (Ott 1993).

### Stem Rot Tolerance Experiment

For the second experiment, the plant material was evaluated, and the results from an *in vitro* assay were kindly provided by K. D. Bowman. The crosses were as follows: Sunki x FD and Pearl x FD. Forty two progeny from the former and 49 from the latter

Table 4.2. *Phytophthora* spp. inoculation dates.

Crosses	Isolates	Dates	
		Inoculation	Scoring
9518, 9519, 9524	Pp101	Aug 25, 97	Nov 4, 97*
	Pp101	Nov 5, 97	Jan 8, 98*
	Pp101	Nov 24, 97	Mar 17, 98*
9522, 9523	Pn117	Apr 15, 97	Jul 7, 97
	Pn117	Jul 8, 97	Aug 19, 97
	Pp101	Aug 20, 97	Oct 16, 97*
	Pp101	Nov 24, 97	Mar 17, 98*
	Pp101	Mar 19, 98	May 27, 98*
	Pn117	Jul 1, 97	Aug 20, 97
	Pn117	Aug 21, 97	Oct 20, 97
	Pn117	Oct 21, 97	Jan 20, 98
	Pn117	Jan 21, 98	May 3, 98
	Pn117	May 4, 98	Jul 28, 98
	Pn117	Jul 29, 98	Oct 9, 98*
	Pp101	Jun 2, 98	Aug 11, 98*
9648, 9669	Pn117	Nov 19, 97	Mar 5, 98
	Pn117	Mar 9, 98	May 27, 98
	Pn117	May 28, 98	Jul 28, 98
	Pn117	Jul 29, 98	Oct 9, 98*
	Pp101	Jun 2, 98	Aug 11, 98*
	Pp101	Aug 12, 98	Oct 23, 98*

\* Scoring dates were used for statistical analyses.

Table 4.3. *Phytophthora* spp. disease severity rating (after Grimm and Hutchison 1973).

Score	Symptom severity	Plant condition
1	No visible symptoms	No damaged roots, resistant
2	Symptoms on few roots	Tolerant
3	Symptoms on majority of roots and some root loss	Intermediate
4	Heavy symptoms, all roots infected, cortex sloughed from major roots	Susceptible
5	Roots dead or missing	No healthy roots

cross were used for *in vitro* etiolated seedling assay for tolerance to *P. nictitanae* Hall 3 isolate. Etiolated seedling preparation, media and inoculum preparation, and an *in vitro* plant inoculation assay were conducted by K. D. Bowman (Bowman 1990, Bowman 1996, per. com. with K. D. Bowman). Ln transformation values of stem lesion length (mm) scores were calculated. The data were categorized in five groups. The results from this *in vitro* etiolated stem assay were used in the RAPD-PCR for identifying *Phytophthora*-tolerance associated DNA markers (see Chapter 5).

## Results

### Root Rot Tolerance Experiment

The average Pp101 and Pn117 root rot disease infection ratings from experiments conducted with citrus and trifoliate orange (DFG50-7) sexual hybrids in 1995 and 1996 are presented in Table 4.4 and Tables A.1-A.8. Disease severity caused by Pp101 generally greater than that caused by Pn117 across all dates and locations in which the experiments were conducted. Chlamydospore propagule count in soil medium differed in experiment dates after inoculations. Disease scoring results did not follow any consistency from one date to another for a given cross and hybrid (Table 4.4 and Table A.9). There were significant differences ( $P \leq 0.05$ ) among the hybrids and the control plants for their *Phytophthora* spp. tolerance in all of the scoring dates. However, when the statistical analysis was performed on the cross level in each scoring date, differences among cross means were not significantly different ( $P \leq 0.05$ ) (Table 4.4). On Oct 16,

1997, average Pp101 disease scores varied from 1.0 to 3.0 with high variation among the replications (Table A.1). The crosses #9522 and #9523 were not significantly different from each other. DFG50-7 TO was more tolerant than both Carrizo and Cleopatra. On Nov 4, 1997, Pp101 disease severity changed from 1.0 to 3.7 having a wider range of variation among the replications (Table A.2). All crosses, #9518, #9519-21, and #9524, were more susceptible than the controls for root rot tolerance. On Jan 8, 1998, *P.palmivora* susceptibility increased in almost all of the hybrids ranging from 2.3 to 4.4 (Table A.3). Cleopatra was more tolerant to Pp101 than DFG50-7 TO and Carrizo at this scoring date. On Mar 17, 1998, Pp101 inoculation reading varied between 1.7 and 5.0 with high variation among the replications (Table A.4). Neither of the crosses showed more tolerance than the control plants. DFG50-7 and Cleopatra were more tolerant than Carrizo. On May 27, 1998, *P.palmivora* disease severity varied from 2.5 to 4.6 towards susceptibility (Table A.5). Variation among the replications was 0.4-1.2. On Aug 11, 1998, hybrid root rot disease varied from very tolerant (1.5) to very susceptible (4.7) with no variation to high degree of variation among the replications (Table A.6). All of the crosses, #9522, #9523, #9648, and #9669, were more susceptible than the control plants. On the last Pp101 scoring date, Oct, 23, 1998, all hybrids, DFG50-7 TO, and Carrizo were more susceptible than Cleopatra (Table A.7). This was the most destructive inoculation period of all.

After several attempts with *P.nicotianae* Pn117 isolate, inoculation dates were not able to differentiate the root rot tolerance among the hybrids, except on Oct 9, 1998 (Table A.8). The hybrids varied from 1.0 to 4.7 for their root rot tolerance in favor of

tolerance. Variation among the replications was very large. Neither of the crosses could be distinguished from another for Pn117 tolerance (Table 4.4). All crosses, #9522, #9523, #9648, and #9669, were more susceptible than the control plants. Carrizo and Cleopatra showed more *P.nicotianae* tolerance than DFG50-7 TO.

Average Pp101 and Pn117 root rot disease infection ratings were calculated for each inoculation date with citrus + citrus and citrus + trifoliate orange somatic hybrids (Table 4.5). After Pp101 inoculation, somatic hybrids had a range of variation from little symptoms in Changsha + Benton (Oct 16, 1997), 'Marsh' grapefruit + Argentine TO (Nov 4, 1997), 'Marsh' grapefruit + rough lemon 8166 (Jan 8, 1998), and Milam + Kinkoji (Oct 23, 1998) to no healthy root systems in sour orange + DFG50-7 TO (Oct 23, 1998). By contrast, all somatic hybrids tested with Pn117 were equally tolerant, except sour orange + Benton.

Correlation of disease rating among different inoculation dates was not highly significant (Table A.10). Relatively high correlation was found between Mar 17, 1998 and May 27, 1998 Pp101 inoculations (22.22 %). Since the fact that individuals in families did not show consistent phenotype expression from one date to the next, orthogonal contrasts were made to compare cross and control means in different dates (Table A.11). Although crosses were not significantly different than each other, all of the crosses were more susceptible to Pp101 than DFG50-7 TO on Nov 4, 1997, Mar 17, 1998, May 27, 1998, and Aug 11, 1998. Carrizo was more tolerant than the hybrids on Nov 4, 1997, Mar 17, 1998, and Aug 11, 1998. The hybrids were more susceptible than Cleopatra on all inoculation dates, except Oct 16, 1997.

Table 4.4. *Phytophthora palmivora* Pp101 and *P. nicotianae* Pp117 inoculations in citrus and trifoliolate orange sexual hybrids<sup>xy</sup>.

Crosses	Oct 16, 1997	Nov 4, 1997	Jan 8, 1998	Mar 17, 1998	May 27, 1998	Aug 11, 1998	Oct 23, 1998	Oct 9, 1998
	Pp101	Pp101	Pp101	Pp101	Pp101	Pp101	Pp101	Pp117
	10 prop/cm <sup>3</sup>	10 prop/cm <sup>3</sup>	15 prop/cm <sup>3</sup>	20 prop/cm <sup>3</sup>	15 prop/cm <sup>3</sup>	5 prop/cm <sup>3</sup>	30 prop/cm <sup>3</sup>	45 prop/cm <sup>3</sup>
	P<0.0205	P<0.0001	P<0.0350	P<0.0001	P<0.0013	P<0.0001	P<0.0001	P<0.0001
9518	-	2.0±0.4 a	3.5±0.6 a	3.6±0.7 a	-	-	-	-
9519-21	-	2.1±0.8 a	3.7±0.6 a	3.5±1.0 a	-	-	-	-
9522	2.0±0.5 a	-	-	3.0±0.9 ab	3.6±0.6 a	3.3±0.7 a	-	1.6±1.0 bc
9523	1.6±0.5 ab	-	-	3.4±0.6 ab	3.5±0.4 a	3.0±0.7 a	-	1.5±0.8 bc
9524	-	2.1±0.5 a	3.6±0.3 a	3.0±0.8 ab	-	-	-	-
9648	-	-	-	-	-	3.0±0.6 a	4.4±0.5 a	1.7±0.7 b
9669	-	-	-	-	-	3.2±0.4 a	4.1±0.5 ab	1.6±0.5 bc
DFG50-7 TO	1.1±0.3 b	1.0±0.0 b	3.4±1.2 a	1.5±0.7 cd	2.3±0.9 b	2.2±0.7 b	3.8±0.8 ab	1.0±0.0 c
Carrizo	1.9±1.2 a	1.3±0.8 b	3.8±1.2 a	2.3±0.6 bc	2.8±1.0 ab	2.5±0.5 b	3.6±0.5 b	3.4±1.1 a
Cleopatra	1.9±1.0 a	1.3±0.5 b	2.7±0.8 b	1.0±0.0 d	2.9±1.1 ab	2.0±0.6 b	2.2±0.8 c	3.8±1.1 a

<sup>z</sup> Average root rot disease scoring from hybrids.<sup>y</sup> Means with the same letter in the same column are not significantly different using Duncan's Multiple Range test, P<0.05.

Table 4.5. *Phytophthora palmivora* Pp101 and *P. nicotianae* Pn117 root rot disease infection rating of citrus and trifoliolate orange somatic hybrids<sup>a,b</sup>.

Plants	Oct 16, 1997 <sup>a</sup>			Nov 4, 1997 <sup>a</sup>			Jan 8, 1998 <sup>a</sup>		
	Pp101, 10 prop/cm <sup>3</sup> P<0.0371	Range N	Means <sup>b</sup>	Pp101, 10 prop/cm <sup>3</sup> P<0.0001	Range N	Means	Pp101, 15 prop/cm <sup>3</sup> P<0.0001	Range N	Means
'Navel' + DFG50-7	1-3	10	1.3±0.7 ab	1-4	10	1.5±1.1 b-d	3-5	10	4.1±0.7 a
Milam + Kinkoji	-	-	-	-	-	-	-	-	-
SO + Benton	-	-	-	-	-	-	-	-	-
'Nova' + 'HBp'	-	-	-	-	-	-	-	-	-
'Hamlin' + RL <sup>a</sup>	-	-	-	-	-	-	-	-	-
SO + Rangpur <sup>a</sup>	1-4	11	1.5±0.9 ab	-	-	-	-	-	-
Changsha + Benton	1-1	8	1.0±0.0 b	-	-	-	-	-	-
Milam + Carrizo	1-2	10	1.6±0.5 ab	-	-	-	-	-	-
Cleopatra + RL8166	-	-	-	1-3	10	2.1±0.6 ab	2-3	9	2.3±0.5 cd
'Succari' + RL8166	-	-	-	1-3	10	2.2±0.6 a	2-3	10	2.3±0.5 cd
'Marsh' + ArgentineTO	-	-	-	1-2	9	1.1±0.3 cd	1-3	9	1.6±0.7 de
'Marsh' + RL8166	-	-	-	1-2	10	1.7±0.5 a-c	1-2	7	1.3±0.5 e
SO + DFG50-7	-	-	-	-	-	-	-	-	-
Grpft + DFG50-7	-	-	-	-	-	-	-	-	-
'Valencia' + 'Femminello'	-	-	-	-	-	-	-	-	-
SO	-	-	-	-	-	-	-	-	-
DFG50-7	1-2	12	1.1±0.3 b	1-1	10	1.0±0.0 d	1-5	10	3.4±1.2 ab
Carrizo	1-4	14	1.9±1.2 a	1-3	6	1.3±0.8 cd	2-5	6	3.8±1.2 a
Cleopatra	1-4	15	1.9±1.0 a	1-2	10	1.3±0.5 cd	1-4	10	2.7±0.8 bc

Table 4.5. cont.

Plants	Pp101			Pp101			Pn117		
	Aug 11, 1998 <sup>w</sup>			Oct 23, 1998 <sup>x</sup>			Oct 9, 1998 <sup>x</sup>		
	P<0.0001, 5 prop/cm <sup>3</sup>			P<0.0001, 30 prop/cm <sup>3</sup>			P<0.0001, 45 prop/cm <sup>3</sup>		
	Range	N	Means	Range	N	Means	Range	N	Means
'Navel' + DFG50-7	2-4	10	3.2±0.6 bc	3-5	10	4.5±0.7 ab	1-2	8	1.1±0.4 c
Milam + Kinkoji	2-4	10	3.1±0.7 bc	1-2	9	1.4±0.5 g	1-1	10	1.0±0.0 c
SO + Benton	2-3	10	2.8±0.4 cd	1-5	10	2.4±1.5 ef	2-3	10	2.3±0.5 b
'Nova' + 'HBP'	3-4	9	3.4±0.5 ab	1-2	9	1.6±0.5 fg	1-2	12	1.3±0.5 c
'Hamlin' + RL <sup>u</sup>	-	-	-	-	-	-	1-2	9	1.1±0.3 c
SO + Rangpur <sup>d</sup>	-	-	-	-	-	-	1-2	8	1.1±0.4 c
Changsha + Benton	3-5	8	3.8±1.0 a	2-5	3	3.3±1.5 cd	-	-	-
Milam + Carrizo	-	-	-	1-3	11	2.4±0.7 e-g	-	-	-
Cleopatra + RL8166	-	-	-	-	-	-	-	-	-
'Succari' + RL8166	-	-	-	-	-	-	-	-	-
'Marsh' + Argentine TO	-	-	-	-	-	-	-	-	-
'Marsh' + RL8166	-	-	-	-	-	-	-	-	-
SO + DFG50-7	-	-	-	5-5	9	5.0±0.0 a	-	-	-
Grpft + DFG50-7	-	-	-	2-5	10	3.5±1.0 cd	-	-	-
'Valencia' + 'Femminello'	-	-	-	3-4	10	3.5±0.5 cd	-	-	-
SO	-	-	-	2-4	10	2.6±0.7 de	-	-	-
DFG50-7	1-3	12	2.3±0.6 de	3-5	5	3.8±0.8 bc	1-1	14	1.0±0.0 c
Carrizo	2-3	11	2.5±0.5 de	3-4	5	3.6±0.5 bc	2-5	13	3.4±1.1 a
Cleopatra	1-3	11	2.0±0.6 e	1-3	5	2.2±0.8 e-g	2-5	18	3.8±1.1 a

<sup>z</sup> Disease scoring: 1: no damaged roots, 5: no healthy roots.

<sup>y</sup> Scores are average of at least three replicate plants.

<sup>w</sup> Maintained in the greenhouse.

<sup>x</sup> Maintained in the growth room.

<sup>y</sup> Means with the same letter in each column are not significantly different using Duncan's Multiple Range test,  $P < 0.05$ .

<sup>u</sup> Seedlings were used instead of cuttings.

Hybrids were more susceptible than Cleopatra and Carrizo in the Pn117 inoculation on Oct 9, 1998. Pp101 was highly destructive in the greenhouse during the experiments (data not shown). The hybrids grown in the greenhouse showed more disease tolerance than growth room grown plants with both *Phytophthora* species (author's observation).

The Pp101 root rot disease scores of the Sunki x FD hybrids were between 1.8 and 3.9, and of the Pearl x FD hybrids were between 2.0 and 4.7 (Table 4.5). Both of the crosses were more susceptible to root rot than Swingle. Overall, the Sunki x FD hybrids were more tolerant than the Pearl x FD hybrids. A relatively high ( $r^2=39.7\%$ ) correlation between Jan 25, 1999 and Apr 13, 1999 inoculation dates (Table A.10). Both crosses were more susceptible than Swingle for their Pp101 root rot disease (Table A.11).

#### Stem Rot Tolerance Experiment

The *in vitro* grown seedling stem disease ratings were extracted from an experiment conducted with *P.nicotianae* Hall 3 isolate (Table 4.6). Ln of stem lesion lengths varied from 1.9 to 3.6 in the Sunki x FD cross, and that varied from 1.6 to 3.5 in the Pearl x FD cross. The Sunki x FD and Pearl x FD hybrids were more susceptible than FD and Swingle. Carrizo suffered from disease pressure to *in vitro* stem inoculations. There was a reasonably high correlation between *in vitro* tests with Pn117 (Sep 30, 97) and *in vivo* responses to Pp101 (Apr 13, 99) ( $r^2=36.4\%$ ) the Pearl x FD cross (Table A.10). Both crosses were more susceptible than Swingle (Table A.11). The hybrids exhibited continuous variation for *P.nicotianae* Hall 3 stem rot symptoms (Fig. 4.1).

Table 4.6. Mean disease scores of *Phytophthora palmivora* Pp101 and *P. nicotianae* Hall3 inoculations with citrus and trifoliate orange (Flying Dragon) hybrids.

Crosses	Pp101 <sup>z</sup>			Hall 3 <sup>y</sup>		
	Range	N	Mean	Range	N	Mean
Sunki x FD	1.8 - 3.9	25	2.8±0.1 b	1.9 - 3.6	42	2.7±0.3
Pearl x FD	2.0 - 4.7	25	3.6±0.1 a	1.6 - 3.5	49	2.6±0.5
FD	-	-	-	-	21	1.7±0.4
Swingle	1.0 - 3.0	25	1.7±0.2 c	-	28	1.9±0.2
Carrizo	-	-	-	-	22	3.1±0.3

<sup>z</sup> Disease scoring: 1: no damaged roots, 5: no healthy roots. Means associated with standard errors were separated by Duncan's Multiple Range Test within each column.  $P \leq 0.05$ . Pp101 inoculation dates, 1/24/99 and 4/13/99, were analyzed together.

<sup>y</sup> Mean Ln of stem lesion length associated with standard error of *P. nicotianae* Hall 3 inoculation provide by K.D.Bowman. Hall 3 inoculation dates were 11/22/96, 4/22/97, 8/1/97, and 9/30/97.

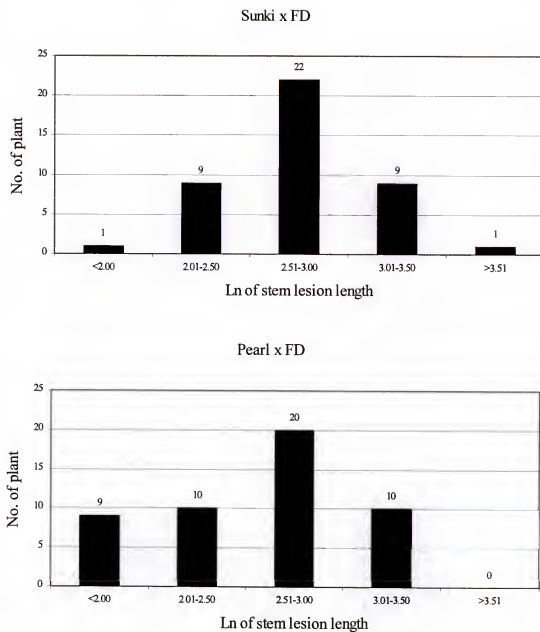


Figure 4.1. Number of hybrids in each disease scoring groups of Sunki x FD and Pearl x FD crosses.

The stem rot disease scores among Sunki x FD and Pearl x FD hybrids were normally distributed.

### Discussion

This was the first screening study for *P.palmivora* and *P.nicotianae* disease tolerance using sexual and somatic hybrids of citrus and trifoliate orange. After 18 different inoculation dates, the mode of inheritance of *Phytophthora* tolerance could not be determined in the present research either in citrus or its close relative, trifoliate orange. One of the reasons for this failure is that a reliable and consistent disease screening technique has not yet been developed. There was large variation among the replicated cuttings of the hybrid seedlings. Exploring host resistance and evaluating hybrid plants for their disease tolerance classification still remain challenge for *Phytophthora* tolerance breeding in citrus hybrids. Another reason is having a relatively small hybrid populations that should be considered. Increasing progeny number and observing variation among the hybrids with environment independent plant screening methods will advance the understanding of the disease resistance mechanism in this host-parasite interaction. The *P.nicotianae* Hall 3 stem rot disease results showed a normally distributed variation among the hybrids from Sunki x FD and Pearl x FD crosses (Fig. 4.1). This may indicate that the tolerance to *P.nicotianae* is controlled by multiple gene regions.

‘Ridge Pineapple’ sweet orange, ‘Gou Tou’ sour orange hybrid, ‘Wainwright’ pummelo, ‘Siamese sweet’ pummelo, ‘Duncan’ grapefruit, LB#6-1 hybrid, and selection #8730 x trifoliate orange DFG50-7 selection cross hybrid seedlings were more

susceptible to Pp101 than Pn117. These non-fruit-bearing young seedling results using two *Phytophthora* species are in agreement with previous studies (Graham 1990, 1995a, 1995b, 1998). In his survey in east, west, and south citrus growing regions of Florida, Graham (1998) observed that both soil conditions and other microorganism activities enhanced *P. palmivora* infections. Trees grown on Swingle and Carrizo showed more severe decline than the trees on Cleopatra, sour orange, and Volkamer lemon. However, Cleopatra and sweet orange performed worse than sour orange, Carrizo, Volkamer lemon, and Swingle under *P. nicotianae* disease pressure. When the results of this current study were compared with Graham's (1995b) findings, similar trends could be found in *P. nicotianae* tolerance among citrus rootstocks. While Cleopatra and 'Gou Tou' were susceptible, Carrizo, *Citrus obovoidea* (Kinkoji), and sour orange were intermediate, and Swingle and DFG50-7 TO were tolerant to *P. nicotianae*. This result shows that *P. palmivora* and *P. nicotianae* have different host infection range on citrus and the resistance mechanisms to each pathogen are under different genetic control.

Surprising results came from sour orange + trifoliate orange somatic hybrid combination. This was expected to be one of the best tolerant hybrid combinations expected since both parents are considerably tolerant to *Phytophthora* species (Graham 1995b, Gmitter *et al.* 1992). When we considered the disease tolerance contribution from sour orange and DFG50-7 TO parents to the somatic hybrids, DFG50-7 TO did play an important role for *P. nicotianae* tolerance, but not for *P. palmivora* tolerance. Sour orange and trifoliate orange have been classified as moderately tolerant and tolerant rootstocks, respectively. Epistatic gene effects may play a role at the tetraploid level. Different genes

may be responsible for tolerance to these two *Phytophthora* spp. Alternatively, the response to *Phytophthora* species exclusion might be accounted for by the morphology of the root system of the somatic hybrids, rather than some direct genetic contribution.

Environmental conditions, i.e. temperature, humidity, light intensity, might have had an impact on the expression of disease tolerance mechanism of the hybrids.

Chlamydospore inoculum density fluctuated in the soil after the inoculations. However, disease severity did not follow the number of propagules existed in soil medium.

Uncontrollable and unfavorable environmental condition might cause *P.palmivora* spores to lose their ability to infect in some of the experiment dates. The *P.nicotianae* Blanton, Wood, and Pn117 isolates were not infectious enough to differentiate the root rot tolerance of hybrid seedlings. Comparison of greenhouse vs. growth room environments was less important during the course of experiments. Regression analysis and orthogonal contrasts of cross means did not find consistent correlation between different experiment times, and cross and control plant means, respectively.

Besides environmental conditions, citrus (evergreen) and trifoliate orange (deciduous) have different growth stages. Dormancy in trifoliate orange in cooler growing seasons can cease the root growth which might be observed in its progeny. The disease severity of *Phytophthora* spp. can vary according to plant growth stage as well. Fluctuation among the disease tolerance results of the hybrids from one inoculation date to another may be explained by the hybrids dormancy condition.

The data acquired from *in vitro* tests from Sunki x FD and Pearl x FD crosses provided enough quantitative measurement information for *P.nicotianae* Hall 3 tolerance.

If the population size was larger, more variation among seedlings may have been observed, leading to easier isolation of the individuals at the resistant extreme for stem rot tolerance. *In vitro* screening experiments need to be conducted with more citrus and other trifoliate orange hybrid selections. Then, the disease screening of promising hybrids need to be repeated in the greenhouse in order to determine whether two methods provide reinforcing results. The caution needs to be taken for relying on *in vitro* selection of disease tolerant individuals whose disease tolerance response might be different in the greenhouse or field tests (Bowman 1990).

Different isolates and inoculum levels should be considered for future root and stem rot screening in the greenhouse experiments. Disease severity rating (Grimm and Hutchison 1973) might be modified to a 1 to 10 scale in order to observe more variation groups with a larger number of progeny. Mycelia, sporangia, and zoospore mixtures (Furr and Carpenter 1961, Grimm and Whidden 1962) may be used for ensuring the stable and consistent root rot disease results.

The Sunki x FD and Pearl x FD crosses were used to identify the association between *Phytophthora* tolerance gene(s) and RAPD markers in the following chapter.

## CHAPTER 5

### IDENTIFICATION OF RAPD MARKERS ASSOCIATED WITH *ALTERNARIA* AND *PHYTOPHTHORA* DISEASE RESISTANCE

#### Introduction

Molecular marker technology has opened a new era in life sciences, leading to great advances in plant genome research. Combining conventional genetics and breeding efforts with molecular and cell biology systems has been yielding substantial new genetic information. A small amount of material brought into a laboratory can provide great insight and a better understanding of the genetics of organisms, or populations of organisms. Not only can the creation, evolution, growth, and development processes of organisms be better explained, but also the relationship between host and parasite interactions can be better understood.

Using current programming technology, computer software programs have been written to enhance the complex mathematical equations and reduce the computation time. MAPMAKER and JOINMAP are two of the examples which are being used in plant breeding, specifically, in marker-trait association and genome mapping. MAPMAKER/EXP performs simultaneous multipoint linkage analysis for dominant, recessive, and co-dominant markers for backcross ( $BC_1$ ),  $F_2$  and  $F_3$  intercrosses, and sib and self-mated recombinant inbred lines (Lander *et al.* 1987, Lincoln *et al.* 1993a).

MAPMAKER/QTL can map genes that are controlling quantitative traits in  $BC_1$  and  $F_2$  families relative to a genetic linkage map (Lincoln *et al.* 1993b). JOINMAP, in addition to the above populations, deals with a wide range of mapping situations, e.g. haploid (HAP, HAP1) and doubled haploid (DH, DH1, DH2), and cross pollinated (CP) populations (Stam 1993, Stam and van Ooijen.1995). JOINMAP can also combine data derived from different sources into an integrated map with up to 500 markers per linkage group. In most instances, JOINMAP calculates shorter linkage distances than MAPMAKER.

Incorporating traditional breeding efforts with the marker-assisted selection (MAS) technique can provide unprecedented speed in breeding of fruit crops. The time required for transferring a desirable trait from an existing or wild germplasm to the cultivated varieties can be shortened by screening of the progeny in the early growing stage. Further, selection for multiple genetic characteristics can be conducted simultaneously, if suitable markers linked to the relevant genes have been identified. This approach to selection of superior individuals from breeding populations can be very efficient and especially useful to perennial plant breeders. The aim of this chapter was to discover dominant RAPD markers associated with *Alternaria* and *Phytophthora* resistance gene regions.

## Materials and Methods

### Genomic DNA Isolation and Bulk Preparation

The DNA extraction method was modified for total DNA extraction from any age of leaves (GibcoBRL 1996, Deng *et al.* 1996b, Ling 1996, Porebski *et al.* 1997): Leaves were ground in 18 X 150 mm glass test tubes with 2 ml lysis buffer [1% cetyltrimethylammonium bromide (CTAB), 5% povyvinyl pyrrolidone (PVP), 1.4 M NaCl, 20 mM EDTA, 10 mM Tris-HCl (pH 8.0), and 350 mM 2-mercaptoethanol] using BioHomogenizer M133/1281-0 (ESGE-Switzerland) for a few seconds. Samples were transferred into sterile 2.0 ml Eppendorf tubes which were centrifuged at 12000 rpm for 5 min in cold. The supernatant (700 µl) was transferred to sterile 1.5 ml Eppendorf tubes. Equal volume (700 µl) of phenol:chloroform:iso-amyl-alcohol (25:24:1) was added to the tubes. Then, the samples were centrifuged at 12000 rpm for 5 min in cold. The supernatant (600 µl) was transfer to sterile 1.5 µl Eppendorf tubes. Equal volume (600 µl) of phenol:chloroform:iso-amyl-alcohol (25:24:1) was added to the tubes. The samples were centrifuged at 12000 rpm for 5 min in cold. After that, the supernatant (500 µl) was transferred to sterile 1.5 µl Eppendorf tubes. Equal volume (500 µl) of chloroform was added to the samples. The tubes were centrifuge at 12000 rpm for 5 min in cold. The supernatant (400 µl) was transferred to sterile 1.5 ml Eppendorf tubes. Then, 2.5 volumes (1000 µl) of 95% ethanol was added to the tubes. The samples were precipitated in the -4°C freezer overnight. The next day, the samples were thawed, gently inverted, and centrifuged at 4000 x g for 15 minutes. The supernatant was discarded. The samples were

rinsed with 70% ethanol. DNA pellet was dried at room temperature. The pellet was re-suspended in 100  $\mu$ l 1 M TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). RNase (Sigma-Aldrich Co., St. Louis, MO) was added (1  $\mu$ l of 500 ng/ml), and the samples were incubated at 37°C at least 1 h (Boekel Industries Inc. Model 133000 incubator). DNA concentration was determined by analyzing 5- $\mu$ l sample on a 1 % agarose high EEO gel. DNA samples were adjusted to 50 ng/100  $\mu$ l an equal concentration in 1 M TE buffer.

For *Alternaria* resistance screening, total DNA from five resistant and five susceptible hybrids from the 'Clementine' x LB#8-10 backcross were bulked, namely R<sub>7</sub> and S<sub>7</sub>. Two different crosses were used for *Phytophthora* tolerance screening. Total DNA from five most tolerant and five most susceptible hybrids from the Sunki x FD were bulked, called R<sub>Sunki</sub> and S<sub>Sunki</sub>. Similarly, DNA from five tolerant plants (R<sub>Pearl</sub>) and five susceptible plants (S<sub>Pearl</sub>) were bulked from the Pearl x FD cross. Twenty  $\mu$ l of equilibrated DNA solutions from each hybrid were mixed in a 500  $\mu$ l-Eppendorf tube to construct each bulk, to be used for primer screening.

### RAPD-PCR Screening

RAPD fragments were amplified by using 492 single, random 10-mer oligonucleotide primers from kits A, B, C, D, E, G, H, O, P, Q, R, S, T, U, V, W, X, AA, AB, AC, AD, AE, AF, AG, AH, AI, AJ, AK, AL, AM, AN series (purchased from Operon Technologies, Inc., Alameda, CA), and DNA bulks as template. Each 15  $\mu$ l of PCR reaction mixture contained 20 mM MgCl<sub>2</sub> ficoll/dye, 200  $\mu$ M 10X dNTP mix (Idaho Technologies, Inc., Idaho, ID), 0.8  $\mu$ M 10-mer primer, 1 unit *Taq* polymerase enzyme

(Promega, Madison, WI), 50 ng/ $\mu$ l genomic DNA, and dd sterile H<sub>2</sub>O (Welsh and McClelland 1990, Williams et al. 1990, Ling 1996). The polymerase chain reaction (PCR) was performed in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., Watertown, MA). The PCR amplification program used was: 94°C for 2 min, then 94°C for 1 min, 35°C for 1 min, 72°C for 2 min, for 43 cycles (Ling 1996).

The amplification products were loaded as pairs of resistance/susceptible bulks in adjacent lanes on 1 % high EEO agarose gels (Fisher Scientific Co., Pittsburgh, PA) prepared with 1 X TAE (0.04 M Tris-acetate, 0.001 M EDTA) and poured on to a Model H4 horizontal gel tray (Life Technologies, Inc., Gaithersburg, MD). The gel tray was submerged horizontally in 1 X TAE buffer. Constant electrical current of 175-200 V was supplied (E-C Apparatus Corporation, Holbrook, NY) during 2.5 h gel runs. For staining, either ethidium bromide was added to the agarose gel before the run and the gels were destained after the run, or the gels were directly placed in water trays containing ethidium bromide solution. Amplified RAPD fragments were detected with an ultraviolet light transilluminator (UVP, Inc., San Gabriel, CA) and photographed using the IS-500 gel documentation system (Alpha Innotech Corporation, San Leandro, CA). The score of '1' for each present fragment and the score of '0' for each absent fragment were assigned. The blank lanes were designated with '-.'

In order to avoid repetition, both *Alternaria* resistance and *Phytophthora* tolerance screening tests were presented together. In the first primer test, resistant (R) and susceptible (S) bulks from 'Clementine' x LB#8-10, Sunki x FD, and Pearl x FD crosses were screened side-by-side with 492 RAPD primers. Because the primers that showed

both faint and strong fragments were chosen, the second round of screening was thought to be necessary for eliminating the fragments which were amplified by mismatching. In the second screening, 63 primers for 'Clementine' x LB#8-10 backcross, and 47 primers for Sunki x FD and Pearl x FD crosses including their parents were repeated. Only 9 primers from the former group and 19 from the latter produced clear and scorable polymorphisms between paired bulk samples. For the third and last screening with PCR, bulks, parents, and the corresponding hybrids were run together. Pearl x FD cross gave more polymorphic fragments (44 markers from 19 primers) than the Sunki x FD (28 markers from 18 primers) and 'Clementine' x LB#8-10 crosses (11 markers from 7 primers). After the third screening, all readable polymorphic fragments were scored, and used in the Chi-square ( $X^2$ ) analysis to test the goodness of fit. MAPMAKER (MAPMAKER/EXP version 3.0b and MAPMAKER/QTL version 1.1b [A Whitehead Institute for Biomedical Research Technical Report, Third Ed., Cambridge, MA]) and JOINMAP (version 2.0/a, CPRO-DCO, Wageningen, the Netherlands) computer programs were used to determine the linkage among the markers. The Kosambi mapping function with minimum 3.0 LOD score and 0.35 linkage ratio were used.

## Results

### RAPD Marker Analysis and Localized Genome Mapping in 'Clementine' x LB#8-10 Backcross for *Alternaria alternata* Resistance

*Alternaria* disease resistance was considered to be a recessive genetic trait based on results from Chapter 3. RAPD fragments were treated as dominant markers. Both MAPMAKER/EXP v3.0b and JOINMAP v2.0 gave very similar results. Table 5.1 presents the JOINMAP v2.0 (JM) linkage analysis results. A total of 6 markers from 5 primers were found to be associated with *Alternaria* resistance in 89 hybrids of the 'Clementine' x LB#8-10 backcross. The Chi-square test showed that AL3<sub>1250</sub> and P12<sub>850</sub> (Fig. 5.1) segregated in a 3:1 ratio (66:23 and 61:28,  $X^2=0.03$  and 1.98, respectively,  $P \leq 0.05$ ). AN6<sub>650</sub>, V3<sub>1500</sub>, S9<sub>800</sub>, and AL3<sub>1600</sub> showed 1:1 segregation ( $X^2=1.90, 2.91, 1.36$ , and 1.36, respectively,  $P \leq 0.05$ ).

Two markers, AL3<sub>1250</sub> and P12<sub>850</sub>, and the putative dominant gene locus, named *AaMI*, were linked to susceptibility, that covered 36.7 cM. No interference between the markers was presumed. Although these two RAPD markers were linked to the *AaMI* locus, the genetic distances were far apart. In the second linkage group, the genetic distance between markers AN6<sub>650</sub> and V3<sub>1500</sub> was 5.7 cM. In the third linkage group, the markers S9<sub>800</sub> and AL3<sub>1600</sub> were 6.7 cM map distance apart. The other markers, S9<sub>1000</sub>, D12<sub>600</sub>, E16<sub>550</sub>, P12<sub>650</sub>, and AN6<sub>900</sub> were not linked.

RAPD Marker Analysis and Linkage Groups in Sunki x FD and Pearl x FD Crosses for *Phytophthora nicotianae* Tolerance

Twelve markers (amplified by 10 primers) formed 6 linkage groups that were associated with *Phytophthora* tolerance in the Sunki x FD cross (Table 5.2). Skewed segregation from 1:1 ratio was observed in linkage group 2 (AJ18<sub>1000</sub> and X18<sub>1000</sub>,  $X^2=5.49$ ), and in linkage group 3 (AC19<sub>1300</sub> and E16<sub>2200</sub>,  $X^2=15.24$  and  $5.26$ , respectively) ( $P \leq 0.05$ ) (Fig. 5.2). While FD had all the markers, Sunki did not carry any of the markers. The genetic mapping distances between the most closely linked markers were follows: 2.4 cM (AA11<sub>2100</sub> and AA11<sub>200</sub>), 4.9 cM (AJ18<sub>1000</sub> and X18<sub>1000</sub>), and 5.7 cM (AC19<sub>1300</sub> and E16<sub>2200</sub>).

In the Pearl x FD population, 16 markers (amplified by 12 primers) formed 8 linkage groups that were associated with *Phytophthora* resistance. The R<sub>Pearl</sub> bulk and the majority of its resistant hybrids carried the following six segregating markers, AJ18<sub>1000</sub>, X18<sub>1000</sub>, AC19<sub>1300</sub>, AM3<sub>700</sub>, AJ4<sub>600</sub>, and S7<sub>1450</sub>. The susceptible plants in the R<sub>Pearl</sub> bulk varied in their possession of markers from none to 4. While FD had 4 out of 6 markers, Pearl was missing the markers which existed in FD, but was carrying the markers absent in FD. AJ18<sub>1000</sub> and X18<sub>1000</sub> co-segregated. The genetic mapping distances were between the most closely linked markers were as follows: 2.1 cM (C15<sub>650</sub> and C15<sub>700</sub>), 4.2 cM (AH6<sub>800</sub> and AB1<sub>900</sub>), and 4.4 cM (AC19<sub>1300</sub> and AM3<sub>700</sub>) (Table 5.2). The Chi-square values for all *P. nicotianae* tolerance associated markers were not significantly different than 1:1 segregation ratio, except AB1<sub>1250</sub>,  $X^2=4.08$  ( $P \leq 0.05$ ).

When these two population hybrids were combined (41+48=89 hybrids) and analyzed together, 7 markers from 7 primers were found to be associated with *P.nicotianae* tolerance and formed 3 linkage groups. AJ18<sub>1000</sub> and X18<sub>1000</sub> (2.3 cM), and AD10<sub>400</sub> and AB1<sub>400</sub> (14.6 cM) maintained the linkage between them. The genetic distance between E16<sub>2200</sub> and AC19<sub>1300</sub> was 19.5 cM, and that between AC19<sub>1300</sub> and AM3<sub>700</sub> 12.6 cM. In the second linkage group, 3 markers were located. There were no indication for interference between the markers. E16<sub>2200</sub> (36:46,  $X^2=1.22$ ), AD10<sub>400</sub> (43:46,  $X^2=0.10$ ), and AB1<sub>400</sub> (41:48,  $X^2=0.55$ ) fit 1:1 segregation ratio ( $P \leq 0.05$ ).

MAPMAKER/QTL v1.1b computer program was performed with the data in Sunki x FD and Pearl x FD crosses. Fixed-QTLs or peaks could not be found with a LOD score above the threshold (2.0) level (data not presented).

Table 5.1. JOINMAP v2.0 analysis of 'Clementine' x LB#8-10 hybrids for *Alternaria alternata* f.sp. *citri* Shinn resistance.

Groups	Markers	No. of gel fragments			X <sup>2</sup>		cM
		1 <sup>z</sup>	0	-	1:1	3:1	
1	AL3 <sub>1250</sub>	66	23	0	20.78*	0.03	
	P12 <sub>850</sub>	61	28	0	12.24*	1.98	21.4
	AaM1	44	43	2 <sup>y</sup>	0.01	27.68*	15.3
2	AN6 <sub>650</sub>	51	38	0	1.90	14.87*	
	V3 <sub>1500</sub>	52	36	1	2.91	11.88*	5.7
3	S9 <sub>800</sub>	39	50	0	1.36	16.81*	
	AL3 <sub>1600</sub>	39	50	0	1.36	16.81*	6.7
Unlinked	AN6 <sub>900</sub>	54	35	0	4.06*	9.75*	-
	D12 <sub>600</sub>	57	32	0	7.02*	5.69*	-
	E16 <sub>550</sub>	70	18	1	30.73*	0.97	-
	P12 <sub>650</sub>	60	29	0	10.80*	2.73	-
	S9 <sub>1000</sub>	41	48	0	0.55	21.07*	-

<sup>z</sup> Gel fragments 1: present, 0: absent, -: missing data when R=0, S=1.

<sup>y</sup> Two hybrids died before they were tested with *Alternaria*.

\* Significant at  $P \leq 0.05$ .

Table 5.2. JOINMAP v2.0 analyses of Sunki x FD and Pearl x FD hybrids for *Phytophthora nicotianae* Hall 3 tolerance.

Groups	Markers	No. of gel fragments			X <sup>2</sup>		cM
		1 <sup>2</sup>	0	-	1:1	3:1	
Sunki X FD							
1	AA11 <sub>2100</sub>	15	26	0	2.95	2.94	2.4
	AA11 <sub>200</sub>	16	25	0	1.98	4.30*	
2	AJ18 <sub>1000</sub>	28	13	0	5.49*	0.98	4.9
	X18 <sub>1000</sub>	28	13	0	5.49*	0.98	
3	AC19 <sub>1300</sub>	8	33	0	15.24*	0.66	5.7
	E16 <sub>2200</sub>	9	26	6	8.26*	0.01	
4	AD10 <sub>400</sub>	19	22	0	0.22	9.96*	9.8
	AB1 <sub>400</sub>	18	23	0	0.61	7.81*	
5	V4 <sub>1050</sub>	20	21	0	0.02	12.37*	14.6
	AA11 <sub>700</sub>	19	22	0	0.22	9.96*	
6	C15 <sub>500</sub>	18	23	0	0.61	7.81*	14.6
	E15 <sub>500</sub>	18	23	0	0.61	7.81*	
Unlinked	A9 <sub>500</sub>	27	14	0	4.12*	1.83	-
	AA11 <sub>1050</sub>	19	22	0	0.22	9.96*	-
	AA11 <sub>400</sub>	39	2	0	33.39*	8.85*	-
	AD10 <sub>650</sub>	24	17	0	1.20	5.93*	-
	AH6 <sub>800</sub>	24	17	0	1.20	5.93*	-
	AH6 <sub>750</sub>	22	19	0	0.22	9.96*	-
	AJ4 <sub>1450</sub>	17	24	0	1.20	5.93*	-
	AK11 <sub>850</sub>	26	13	2	4.33*	1.44	-
	AK11 <sub>700</sub>	17	22	2	0.64	7.19*	-
	AM3 <sub>700</sub>	17	24	0	1.20	5.93*	-
	B11 <sub>1150</sub>	24	17	0	1.20	5.93*	-
	C15 <sub>900</sub>	24	17	0	1.20	5.93*	-
	E16 <sub>1100</sub>	15	20	6	0.71	5.95*	-
	O5 <sub>1300</sub>	28	13	0	5.49*	0.99	-
	S7 <sub>850</sub>	21	20	0	0.02	12.36*	-
	V4 <sub>800</sub>	24	17	0	1.20	5.93*	-

Table 5.2. continued

Groups	Markers	No. of gel fragments			X <sup>2</sup>		cM
		1 <sup>2</sup>	0	-	1:1	3:1	
Pearl X FD							
1	AJ18 <sub>1000</sub>	29	19	0	2.08	5.44*	0.0
	X18 <sub>1000</sub>	29	19	0	2.08	5.44*	
2	C15 <sub>650</sub>	25	22	1	0.19	12.20*	2.1
	C15 <sub>700</sub>	26	21	1	0.53	8.49*	
3	AH6 <sub>800</sub>	28	20	0	1.33	7.11*	4.2
	AB1 <sub>900</sub>	26	22	0	0.33	11.11*	
4	AC19 <sub>1300</sub>	18	30	0	3.00	4.00*	4.4
	AM3 <sub>700</sub>	17	29	2	3.13	3.51	
5	X18 <sub>500</sub>	20	28	0	1.33	7.11*	10.4
	AB1 <sub>1250</sub>	17	31	0	4.08*	2.78	
6	AJ4 <sub>600</sub>	23	25	0	0.04	13.44*	14.6
	S7 <sub>1450</sub>	20	28	0	1.33	7.11*	
7	AD10 <sub>400</sub>	24	24	0	0.00	16.00*	18.8
	AB1 <sub>400</sub>	23	25	0	0.53	13.44*	
8	U4 <sub>550</sub>	24	24	0	0.00	16.00*	18.8
	O5 <sub>650</sub>	21	27	0	0.75	9.00*	
Unlinked	A9 <sub>900</sub>	13	35	0	10.08*	0.11	-
	A9 <sub>850</sub>	23	25	0	0.08	13.44*	-
	A9 <sub>650</sub>	30	18	0	3.00	4.00*	-
	A9 <sub>500</sub>	27	21	0	0.75	9.00*	-
	A9 <sub>200</sub>	24	24	0	0.00	16.00*	-
	AA11 <sub>1050</sub>	20	28	0	1.33	7.11*	-
	AA11 <sub>550</sub>	21	27	0	0.75	9.00*	-
	AA11 <sub>400</sub>	24	24	0	0.00	16.00*	-
	AB1 <sub>850</sub>	26	22	0	0.33	11.11*	-
	AH6 <sub>400</sub>	24	24	0	0.00	16.00*	-
	AJ4 <sub>1450</sub>	22	26	0	0.33	11.11*	-
	AK11 <sub>850</sub>	24	24	0	0.00	16.00*	-
	AK11 <sub>700</sub>	23	25	0	0.08	13.44*	-
	AK11 <sub>550</sub>	31	17	0	4.08*	2.77	-
	AK11 <sub>400</sub>	22	26	0	0.33	11.11*	-

Table 5.2. continued

Groups	Markers	No. of gel fragments			X <sup>2</sup>		cM
		1 <sup>z</sup>	0	-	1:1	3:1	
Pearl X FD							
Unlinked	AM3 <sub>550</sub>	27	19	2	1.39	6.52*	-
	B11 <sub>1150</sub>	17	31	0	4.08*	2.77	-
	C15 <sub>900</sub>	24	23	1	0.02	14.36*	-
	C15 <sub>500</sub>	24	23	1	0.02	14.36*	-
	E16 <sub>2200</sub>	27	20	1	1.04	7.72*	-
	E16 <sub>1100</sub>	20	27	1	1.04	7.72*	-
	H3 <sub>600</sub>	31	17	0	4.08*	2.77	-
	O5 <sub>600</sub>	26	22	0	0.33	11.11*	-
	O5 <sub>400</sub>	20	28	0	1.33	7.11*	-
	O5 <sub>300</sub>	23	25	0	0.08	13.44*	-
	S7 <sub>1350</sub>	28	20	0	1.33	7.11*	-
	S7 <sub>850</sub>	21	27	0	0.75	9.00*	-
	V4 <sub>1050</sub>	25	23	0	0.08	13.44*	-
Sunki and Pearl X FD							
1	AJ18 <sub>1000</sub>	57	32	0	7.02*	5.70*	2.3
	X18 <sub>1000</sub>	57	32	0	7.02*	5.70*	
2	E16 <sub>2200</sub>	36	46	6	1.22	15.63*	19.5
	AC19 <sub>1300</sub>	26	63	0	15.38*	0.84	
	AM3 <sub>700</sub>	34	53	2	4.15*	9.20*	
3	AD10 <sub>400</sub>	43	46	0	0.10	25.80*	14.6
	AB1 <sub>400</sub>	41	48	0	0.55	21.07*	
Unlinked	A9 <sub>500</sub>	54	35	0	4.06*	9.75*	-
	AA11 <sub>1050</sub>	39	50	0	1.36	16.81*	-
	AA11 <sub>400</sub>	63	26	0	15.38*	0.84	-
	AH6 <sub>800</sub>	48	41	0	0.55	21.07*	-
	AJ4 <sub>1450</sub>	39	50	0	1.36	16.81*	-
	AK11 <sub>850</sub>	40	47	2	0.56	20.41*	-
	AK11 <sub>700</sub>	40	49	0	0.91	18.88*	-
	C15 <sub>900</sub>	48	40	1	0.73	19.64*	-
	C15 <sub>500</sub>	42	46	1	0.18	24.24*	-
	E16 <sub>1100</sub>	35	47	7	1.76	13.68*	-
	V4 <sub>1050</sub>	45	44	0	0.01	28.35*	-

<sup>z</sup> Gel fragments 1: present, 0: absent, -: missing data when R=1, S=0.

\* Significant at  $P \leq 0.05$ .

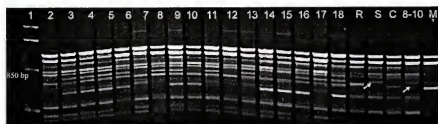


Figure 5.1. RAPD-PCR pattern of the 'Clementine' x LB#8-10 backcross hybrids with the marker P12<sub>850</sub> associated with *Alternaria* susceptibility. The first column from left in the gel is a 1 Kb DNA size marker. The columns from 2 to 18 are the hybrids, and the columns from 19 to 23 are resistant bulk, susceptible bulk, 'Clementine', LB#8-10, and 'Minneola,' respectively.

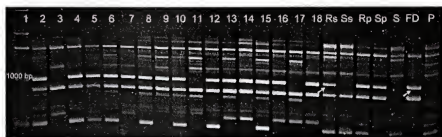


Figure 5.2. RAPD-PCR pattern of the Sunki x FD and Pearl x FD hybrids with the marker X18<sub>1000</sub> associated with *Phytophthora* tolerance. The first column from left in the gel is a 1 Kb DNA size marker. The columns from 2 to 18 are the hybrids, and the columns from 19 to 25 are resistant Sunki x FD bulk, susceptible Sunki x FD bulk, resistant Pearl x FD bulk, susceptible Pearl x FD bulk, Sunki, FD, and Pearl, respectively.

### Discussion

If it is absence of the dominant allele that confers *Alternaria alternata* f.sp. *citri* Shinn resistance, this dominant allele, *AaMI*, from LB#8-10 (Ss) through ‘Minneola’ (SS) conferring brown spot susceptibility is in a coupling phase with AL3<sub>1250</sub> and P12<sub>850</sub> RAPD-PCR markers. In other words, disease resistance conferring recessive allele for *Alternaria* resistance from ‘Clementine’ (ss) is in repulsion with AL3<sub>1250</sub> and P12<sub>850</sub> markers. Making selection against AL3<sub>1250</sub> and P12<sub>850</sub> markers might increase the efficiency of the selection having more homozygous recessive individuals in the population (Haley 1994a, Kelly 1995). Furthermore, markers associated with the resistance allele would be useful to identify heterozygous susceptible individuals, which are more valuable as breeding parents for resistance as well. Relatively long marker distances should be noticed for plausible crossing over events. However, it is simpler and less expensive to screen for *Alternaria* resistance by direct challenge with conidia than by marker-assisted selection (MAS).

A possible cause of the skewed marker segregation ratios in the ‘Clementine’ x LB#8-10 backcross (89), and Sunki x FD (41) and Pearl x FD (48) F<sub>1</sub>-type crosses could be explained by the relatively small number of hybrids in the families. Skewed segregation ratios were observed in other intergeneric citrus backcrosses (Durham *et al.* 1992, Cai *et al.* 1994, Weber 1999). Non-neutral DNA marker loci containing coding and non-coding sequences could be under negative or positive selection pressure. Crossing over interference at the centromeric or telomeric regions was speculated.

Although citrus and trifoliate orange are two closely related genera, there are enough genomic differences for discovering more scorable RAPD-PCR markers in their intergeneric  $F_1$  cross hybrids (Cai *et al.* 1994, Ling 1996, Gmitter *et al.* 1996, Luro *et al.* 1996, Deng *et al.* 1997) for *Phytophthora* tolerance than for the more closely related intra-*Citrus* crosses studied for *Alternaria* resistance. If the number of hybrids were greater, and their disease resistance ratings were sufficiently accurate to prepare the bulks, the finding more associated markers would be more likely. Moreover, more hybrids at each extreme can be included in plant DNA bulk preparation. However, disease resistance phenotyping remains the greatest challenge for studies of resistance to *Phytophthora* (Klotz *et al.* 1958a, Broadbent *et al.* 1971, Grimm and Hutchison 1977, Graham 1995b).

The RAPD primers AL3 associated with *Alternaria* resistance and X18 associated with *P. nicotianae* tolerance were located at the same linkage group with *Ctrv* resistance gene in *Citrus* genome map (Ling *et al.* 1999). These results give promise to the proposed disease resistant gene clusters hypothesis that different resistance genes or functional alleles can be found close to each other at the same genomic regions (Pryor 1987, Keen 1990). Nonetheless, before the fragments from these resembling primers are sequenced and characterized, the helpfulness of RAPD markers could be still in question for identifying clustered disease resistance genes for above mentioned resistance genes in citrus and trifoliate orange.

The AJ18<sub>1000</sub>, X18<sub>1000</sub>, E16<sub>2200</sub>, AC19<sub>1300</sub>, AM3<sub>700</sub>, AD10<sub>400</sub>, and AB1<sub>400</sub> RAPD markers have been obtained from *in vitro* etiolated stem cutting assay with *Phytophthora*

*nicotianae* Hall 3. These selected markers may be useful, but first the correlation of *in vitro* results with field performance needs to be established before such markers could be used in MAS (Vardi *et al.* 1986, Prabhu and Rush 1997). The high likelihood of polygenic control of *Phytophthora* needs to be taken under consideration while screening populations with these markers. *Phytophthora* tolerance genes could be located in different regions of the *Citrus* and *Poncirus* genomes. Another aspect is that these markers have been generated from F<sub>1</sub> type *Citrus* and *Poncirus* intergeneric cross. The markers which are identified from one population might not be suitable for other populations.

An alternative approach to *Phytophthora* tolerance-linked marker development would be QTL analysis based on interval mapping. Consistent and reliable phenotyping, and fairly well-saturated total genome map of the family with marker would be requirements for interval mapping analysis.

Another idea for future efforts to find markers linked to *Phytophthora* tolerance would be to focus on resistant gene candidate (RGC) sequences, or degenerate primers based on other disease-resistance genes (per. com. with F. G. Gmitter, Jr.). The necessity for finding universal markers associated with *Phytophthora* tolerance still remains a frontier.

## CHAPTER 6 SUMMARY AND CONCLUSION

At the conclusion of this study, useful results have been obtained for future research in citrus disease resistance breeding. The data obtained from 'Clementine' (ss) x LB#8-10 (Ss) backcross supports the hypothesis that homozygous recessive form of *AaM1* (acronym of *Alternaria alternata* 'Minneola' 1) gene confirms *Alternaria* disease resistance. This is the first *Alternaria* disease resistance gene described in citrus. The reciprocal backcross in which paternal cytoplasmic genome contribution could have played a role did not entirely support this conclusion because of skewed segregation ratios. However, skewed segregation ratios have been observed in previously studied citrus and trifoliate orange intergeneric crosses. Increasing the number of progeny can be one way to reduce such skewness. Some other  $F_1$ ,  $F_2$ , and backcross hybrids need to be tested with variable isolates that have been collected around the citrus growing regions from brown spot diseased plants for improving potential horizontal resistance of *Alternaria* disease. Using the detached leaf assay, true prediction for field resistance can be achieved for hybrids in a breeding program.

Only 2 putative RAPD markers, AL3<sub>1250</sub> and P12<sub>850</sub>, were found to be linked in repulsion phase to *Alternaria* recessive resistance allele, *aaM1*. Making selection against P12<sub>850</sub> can be helpful for choosing the homozygous recessive plants that are resistant to

*Alternaria*. Since citrus cultivars have been acquired either from chance seedlings, natural hybridizations, or point mutations, the RAPD marker screening technique is limited in its effectiveness because of the fairly high level of homogeneity within intra-citrus-families. Unless the marker is from the gene itself, it is more feasible to use the detached leaf assay with conidia for direct selection instead of MAS.

The mode of inheritance of *Phytophthora* spp. tolerance was not unequivocally determined in this study. The *Phytophthora* root and stem rot disease tolerance of citrus is not easily evaluated. Plant screening has been a challenge for years for gaining solid information about the genetic control of resistance to this disease. Phenotyping inconsistencies across from replications and inoculation dates associated with the screening procedures were a contributing factor to that failure. Even in the controlled conditions of greenhouse and growth room experiments, inconsistent phenotyping results related, perhaps, to environmental factors that affect plant screening procedures. This suggests that multiple genes with small effects on tolerance control overall disease tolerance. This points out the difficulty of pathogen-based screening for this trait, and the potential value for good markers that could be applied to MAS.

The DFG50-7 selection of trifoliolate orange and Cleopatra mandarin behaved differently under the comparison between and *P.palmivora* and *P.nicotianae* for their tolerance to the disease in these greenhouse and growth room experiments. While DFG50-7 TO and its hybrids were more sensitive to *P.palmivora* and more tolerant to *P.nicotianae*, Cleopatra mandarin showed more sensitivity to *P.nicotianae* than *P.palmivora*. One of the contributions of this research is the existence of different host

ranges in *Phytophthora* spp. DFG50-7 TO and Cleopatra appear to carry different resistance genes in their genome composition against these two different species of *Phytophthora*.

Somatic hybrids gave both promising and surprising results during the course of the experiment period. Although DFG50-7 TO involved somatic hybrids were expected to be more resistant, they showed a more susceptible response than some other citrus somatic hybrids to *P. palmivora*. Maybe the high levels of resistance of somatic hybrids is a consequence of tetraploid morphology, and not related to the genes functioning at the diploid level. Somatic hybrids containing Milam, rough lemon, Cleopatra, and Kinkoji as parents are in rootstock field trials for evaluation of their root and foot rot resistance and other horticultural characteristics in Florida.

The quantitative stem rot measurement from Sunki x FD and Pearl x FD hybrids presented a continuous variation for disease tolerance to *P. nicotianae* during the *in vitro* inoculation tests. There are 7 RAPD markers, AJ18<sub>1000</sub>, X18<sub>1000</sub>, E16<sub>2200</sub>, AC19<sub>1300</sub>, AM3<sub>700</sub>, AB1<sub>400</sub>, and AD10<sub>400</sub>, within 3 linkage groups which gave promising results for future investigations in citrus genome for *Phytophthora* disease resistance gene region(s). In these crosses, more *P. nicotianae* associated markers expected to be found because of the recombination nature of the intergeneric crosses. A relatively small number of progeny is another reason in addition to the lack of confidence on the disease screening. Using dominant RAPD molecular markers with integration of MAS, time consuming efforts can be shortened by conducting many rounds of selection without actual pathogen screening (Mohan *et al.* 1997) for disease resistance breeding. Further, resistance to

multiple pathogens can be selected in simultaneous fashion. MAS can be performed at the early growing stage of hybrid seedlings to select useful breeding material by screening them for more than one characteristic at the same time.

Different sources of germplasm which are sexually compatibly with citrus need to be screened for their *Phytophthora* spp. tolerance, and included in breeding program. Then, hybrids can be exposed to mycelia, chlamydospore, and zoospore mixture with a container inoculation technique for their resistance to *Phytophthora* spp. Environmental conditions, for example, temperature, light, irrigation, and humidity, should be monitored very closely in the greenhouse. A quantitative and consistent disease scoring technique must be established for observing *Phytophthora* spp. tolerance among hybrids. After that, field trials need to be designed before any accession will be released as new rootstocks for the citrus industry.

Disease resistance research is important to citrus breeding programs worldwide. Finding closely linked molecular markers, preferably from the gene itself, to the disease resistance genes which are thought to be clustered in the genome will improve the selection of multiple desirable traits in this genus. The basic information obtained from this research about the inheritance of *Alternaria* and *Phytophthora* disease resistance gene(s) and associated RAPD markers might help to locate disease resistance gene clusters, which already contain the citrus tristeza virus and citrus nematode resistance genes, in citrus and trifoliate orange genomes. Moreover, QTL analysis with internal mapping, RGC sequences, or degenerate primers would be other molecular citrus breeding approaches.

APPENDIX  
*PHYTOPHTHORA* SPP. INOCULATIONS IN CITRUS AND TRIFOLIATE ORANGE  
HYBRIDS

Table A.1. *Phytophthora palmivora* Pp101 root rot disease infection rating of 'Wainwright' pummelo (9522) and 'Siamese' pummelo (9523) x DFG50-7 TO hybrids in the greenhouse on Oct 16, 1997<sup>xy</sup>.

Plants	Range	N	Means	Plants	Range	N	Means
9522.1.1	1-4	7	2.4±1.0 a-d	9523.1.2	1-4	12	1.3±0.6 d-g
9522.1.2	1-4	5	2.6±1.1 a-c	9523.1.3	1-3	12	1.4±0.8 c-g
9522.1.5	1-5	7	1.9±1.6 a-g	9523.1.5	1-3	7	1.4±0.8 c-g
9522.1.6	1-4	5	2.2±1.6 a-g	9523.1.6	-	-	-
9522.1.7	1-4	8	1.9±1.1 a-g	9523.1.7	1-3	6	1.7±1.0 b-g
9522.1.8	-	-	-	9523.1.8	1-2	9	1.2±0.4 d-g
9522.1.9	1-3	10	2.0±0.7 a-g	9523.1.10	-	-	-
9522.1.10	1-4	8	1.9±1.0 a-g	9523.1.11	1-3	7	1.3±0.8 d-g
9522.1.11	1-5	8	2.6±1.8 a-c	9523.1.12	-	-	-
9522.1.12	1-4	8	1.6±1.2 b-g	9523.1.13	1-4	6	1.8±1.3 a-g
9522.1.13	1-5	14	2.3±1.3 a-f	9523.1.14	2-3	7	2.7±0.5 ab
9522.1.15	2-4	6	2.7±1.0 ab	Mean			1.6±0.5
9522.1.17	1-5	12	2.2±1.1 a-g	DFG50-7 TO	1-2	12	1.1±0.3 fg
9522.1.21	1-3	11	1.2±0.6 e-g	Carrizo	1-4	14	1.9±1.2 a-g
9522.1.22	1-1	13	1.0±0.0 g	Cleopatra	1-4	15	1.9±1.0 a-g
9522.1.23	1-4	6	2.3±1.2 a-e				<sup>z</sup> Disease scoring: 1: no damaged roots,
9522.1.24	-	-	-				5: no healthy roots.
9522.2.1	1-5	10	1.8±1.5 a-g				<sup>y</sup> Means associated with standard deviation
9522.2.2	1-4	12	1.6±1.0 b-g				with the same letter are not significantly
9522.2.3	1-4	15	1.7±1.1 b-g				different using Duncan's Multiple Range
9522.2.4	2-5	4	3.0±1.4 a				test, P≤0.05.
9522.2.5	-	-	-				
9522.2.6	1-3	12	1.3±0.6 d-g				
Mean			2.0±0.5				

#### Analysis of Variance

Source	DF	SS	MS	F Value	Pr > F
Model	30	70.1549062	2.3384969	2.26	0.0003
Error	257	265.6228716	1.0335520		
Corrected Total	287	335.7777778			
R-Square		C.V.	Root MSE	Mean	
0.208933		57.18587	1.01664	1.77778	

Table A.2. *Phytophthora palmivora* Pp101 root rot disease infection rating of 'Ridge Pineapple' orange (9518), 'Gou Tou' sour orange hybrid (9519-21), and 'Duncan' (9524) x DFG50-7 TO hybrids in the greenhouse on Nov 4, 1998<sup>a,b</sup>.

Plants	Range	N	Means	Plants	Range	N	Means
9518.3.2	1-5	6	1.8±1.6 a-d	9520.1.3	1-3	5	1.4±0.9 b-d
9518.3.3	1-4	4	2.0±1.4 a-d	9520.1.5	1-2	9	1.3±0.5 cd
9518.4.1	-	-	-	9520.2.2	1-3	7	1.4±0.8 b-d
9518.4.2	1-5	7	3.0±1.9 a-d	9521.1.1	1-5	7	3.3±1.7 a-c
9518.5.1	1-5	4	2.0±2.0 a-d	Mean			2.1±0.8
9518.6.1	1-3	15	1.7±0.8 a-d	9524.1.1	1-4	6	2.2±1.2 a-d
9518.6.2	1-4	7	2.1±1.1 a-d	9524.1.2	1-4	9	2.1±1.3 a-d
9518.6.3	1-5	5	1.8±1.8 a-d	9524.1.4	-	-	-
9518.7.2	1-5	8	2.0±1.6 a-d	9524.2.1	1-5	12	2.4±1.6 a-d
9518.9.1	1-5	8	2.4±1.3 a-d	9524.2.2	1-5	13	1.8±1.1 a-d
9518.11.1	1-4	7	1.7±1.1 a-d	9524.2.4	1-4	6	1.8±1.3 a-d
9518.11.2	1-2	4	1.3±0.5 d	9524.2.5	1-5	4	2.5±1.7 a-d
9518.12.2	1-3	3	2.0±1.0 a-d	9524.2.6	1-4	7	2.3±1.3 a-d
9518.13.1	-	-	-	9524.2.7	1-5	4	2.0±2.0 a-d
9518.13.2	1-4	3	2.0±1.7 a-d	9524.2.8	1-5	5	3.0±1.9 a-d
9518.13.3	1-4	8	1.9±1.1 a-d	9524.2.9	1-1	5	1.0±0.0 d
9518.13.4	1-2	3	1.3±0.6 b-d	9524.2.10	1-4	5	1.8±1.3 a-d
9518.13.5	1-5	7	2.7±1.7 a-d	9524.2.11	1-4	9	1.9±1.3 a-d
9518.13.6	-	-	-	9524.2.12	1-5	4	2.3±1.9 a-d
Mean			2.0±0.4	9524.2.13	-	-	-
9519.1.1	1-2	3	1.3±0.6 b-d	Mean			2.1±0.5
9519.2.1	1-5	5	1.8±1.8 a-d	DFG50-7 TO	1-1	10	1.0±0.0 d
9519.2.4	1-5	3	3.3±2.0 ab	Carrizo	1-3	6	1.3±0.8 b-d
9519.2.5	1-3	11	1.7±0.9 a-d	Cleopatra	1-2	10	1.3±0.5 cd
9519.3.3	-	-	-	<sup>a</sup> Disease scoring: 1: no damaged roots, 5: no healthy roots.			
9519.4.1	1-4	9	1.8±1.2 a-d				
9519.4.2	1-5	6	3.7±2.1 a	<sup>b</sup> Means associated with standard deviation with the same letter are not significantly different using Duncan's Multiple Range test, P≤0.05.			
9519.4.5	1-5	7	1.9±1.6 a-d				
9519.4.6	-	-	-				
9520.1.1	1-4	3	2.0±1.7 a-d				
9520.1.2	1-4	4	1.8±1.5 a-d				

#### Analysis of Variance

Source	DF	SS	MS	F Value	Pr > F
Model	44	94.0886380	2.1383781	1.25	0.1533
Error	246	422.4955544	1.7174616		
Corrected Total	290	516.5841924			
	R-Square	C.V.	Root MSE	Mean	
	0.182136	66.78830	1.31052	1.96220	

Table A.3. *Phytophthora palmivora* Pp101 root rot disease infection rating of 'Ridge Pineapple' orange (9518), 'Gou Tou' sour orange hybrid (9519-21), and 'Duncan' (9524) x DFG50-7 TO hybrids in the growth room on Jan 8, 1998<sup>z,y</sup>.

Plants	Range	N	Means	Plants	Range	N	Means
9518.3.2	1-5	6	2.3±1.6 e	9520.1.3	3-5	5	4.0±0.7 a-d
9518.3.3	3-5	3	3.7±1.2 a-e	9520.1.5	1-5	7	3.6±1.9 a-e
9518.4.1	-	-	-	9520.2.2	4-5	7	4.3±0.5 a-c
9518.4.2	4-5	7	4.4±0.5 a	9521.1.1	2-4	7	3.1±0.7 a-e
9518.5.1	1-5	5	3.0±1.6 a-e	Mean			3.7±0.6
9518.6.1	1-4	15	3.1±0.8 a-e	9524.1.1	2-5	6	4.0±1.1 a-d
9518.6.2	2-5	7	3.7±1.0 a-e	9524.1.2	1-4	9	3.6±1.0 a-e
9518.6.3	4-4	4	4.0±0.0 a-d	9524.1.4	-	-	-
9518.7.2	3-5	8	4.1±0.6 a-d	9524.2.1	2-5	11	4.0±1.0 a-d
9518.9.1	1-4	8	3.1±1.0 a-e	9524.2.2	1-5	12	3.3±1.4 a-e
9518.11.1	3-4	7	3.4±0.5 a-e	9524.2.4	1-4	6	2.8±1.0 b-e
9518.11.2	3-5	4	4.3±1.0 a-d	9524.2.5	3-4	4	3.8±0.5 a-e
9518.12.2	-	-	-	9524.2.6	4-4	7	4.0±0.0 a-d
9518.13.1	3-5	3	4.0±1.0 a-d	9524.2.7	1-5	4	3.5±1.7 a-e
9518.13.2	-	-	-	9524.2.8	3-4	5	3.8±0.4 a-e
9518.13.3	1-5	8	2.8±1.4 c-e	9524.2.9	3-4	6	3.7±0.5 a-e
9518.13.4	3-4	3	3.3±0.6 a-e	9524.2.10	3-5	6	3.8±0.8 a-e
9518.13.5	1-4	6	3.2±1.2 a-e	9524.2.11	2-5	8	3.6±0.9 a-e
9518.13.6	-	-	-	9524.2.12	2-5	3	3.7±1.5 a-e
Mean			3.5±0.6	9524.2.13	-	-	-
9519.1.1	-	-	-	Mean			3.6±0.3
9519.2.1	1-4	5	2.4±1.1 e	DFG50-7 TO	1-5	10	3.4±1.2 a-e
9519.2.4	-	-	-	Carrizo	2-5	6	3.8±1.2 a-e
9519.2.5	2-5	11	3.8±0.9 a-e	Cleopatra	1-4	10	2.7±0.8 de
9519.3.3	-	-	-	<sup>z</sup> Disease scoring: 1: no damaged roots, 5: no healthy roots.			
9519.4.1	1-5	9	3.6±1.4 a-e				
9519.4.2	2-5	5	3.2±1.3 a-e	<sup>y</sup> Means associated with standard deviation with the same letter are not significantly different using Duncan's Multiple Range test, P≤0.05.			
9519.4.5	3-5	8	4.1±0.8 a-d				
9519.4.6	-	-	-				
9520.1.1	3-5	3	4.0±1.0 a-d				
9520.1.2	4-5	3	4.3±0.6 ab				

#### Analysis of Variance

Source	DF	SS	MS	F Value	Pr > F
Model	41	69.4640346	1.6942447	1.57	0.0207
Error	235	253.3879509	1.0782466		
Corrected Total	276	322.8519856			
	R-Square	C.V.	Root MSE	Mean	
	0.215158	29.35031	1.03839	3.53791	

Table A.4. *Phytophthora palmivora* Pp101 root rot disease infection rating of 'Ridge Pineapple' orange (9518), 'Gou Tou' sour orange hybrid (9519-21), and 'Duncan' grapefruit (9524), 'Wainwright' pummelo (9522), and 'Siamese' pummelo (9523) x DFG50-7 TO hybrids in the growth room on Mar 17, 1998<sup>xy</sup>.

Plants	Range	N	Means	Plants	Range	N	Means
9518.3.2	2-5	3	3.7±1.5 a-e	9522.1.1	1-5	3	3.0±2.0 a-f
9518.3.3	-	-	-	9522.1.2	1-5	3	2.7±2.1 a-f
9518.4.1	1-5	5	3.4±1.7 a-f	9522.1.5	2-2	3	2.0±0.0 c-f
9518.4.2	4-5	3	4.7±0.6 ab	9522.1.6	2-5	3	4.0±1.7 a-d
9518.5.1	4-5	4	4.8±0.5 ab	9522.1.7	1-5	3	3.0±2.7 a-f
9518.6.1	3-4	3	3.3±0.6 a-f	9522.1.8	1-3	3	2.0±1.0 c-f
9518.6.2	-	-	-	9522.1.9	3-5	3	4.3±1.2 a-c
9518.6.3	4-5	3	4.7±0.6 ab	9522.1.10	1-5	5	3.8±1.8 a-e
9518.7.2	3-5	3	4.3±1.2 a-c	9522.1.11	2-3	3	2.3±0.6 b-f
9518.9.1	3-4	3	3.7±0.6 a-e	9522.1.12	2-5	3	4.0±1.4 a-d
9518.11.1	3-4	3	3.3±0.6 a-f	9522.1.13	1-5	3	2.3±2.3 b-f
9518.11.2	2-4	3	3.0±1.0 a-f	9522.1.15	1-3	3	1.7±1.2 d-f
9518.12.2	-	-	-	9522.1.17	-	-	-
9518.13.1	1-5	3	3.0±2.0 a-f	9522.1.21	2-4	5	3.2±1.1 a-f
9518.13.2	-	-	-	9522.1.22	3-5	3	4.3±1.2 a-c
9518.13.3	2-3	3	2.3±0.6 b-f	9522.1.23	3-5	3	4.3±1.2 a-c
9518.13.4	2-5	3	3.3±1.5 a-f	9522.1.24	2-3	3	2.3±0.6 b-f
9518.13.5	2-4	3	3.0±1.0 a-f	9522.2.1	3-3	3	3.0±0.0 a-f
9518.13.6	2-4	3	3.3±1.2 a-f	9522.2.2	2-4	3	3.0±1.0 a-f
Mean			3.6±0.7	9522.2.3	2-5	5	3.4±1.1 a-f
9519.1.1	2-5	3	3.0±1.7 a-f	9522.2.4	2-5	5	3.4±1.5 a-f
9519.2.1	2-5	3	3.3±1.5 a-f	9522.2.5	1-2	3	1.7±0.6 d-f
9519.2.4	1-5	3	3.0±2.0 a-f	9522.2.6	-	-	-
9519.2.5	1-3	3	2.3±1.2 b-f	Mean			3.0±0.9
9519.3.3	1-5	3	2.7±2.1 a-f	9523.1.2	3-4	3	3.3±0.6 a-f
9519.4.1	-	-	-	9523.1.3	-	-	-
9519.4.2	1-3	3	2.3±1.2 b-f	9523.1.5	3-5	3	3.7±1.2 a-e
9519.4.5	-	-	-	9523.1.6	4-4	3	4.0±0.0 a-d
9519.4.6	1-5	3	3.0±2.0 a-f	9523.1.7	2-5	3	3.3±1.5 a-f
9520.1.1	3-5	4	4.0±0.8 a-d	9523.1.8	2-4	3	3.0±1.0 a-f
9520.1.2	4-5	3	4.7±0.6 ab	9523.1.10	2-5	3	3.3±1.5 a-f
9520.1.3	-	-	-	9523.1.11	2-3	3	2.3±0.6 b-f
9520.1.5	5-5	3	5.0±0.0 a	9523.1.12	2-5	3	4.0±1.7 a-d
9520.2.2	5-5	3	5.0±0.0 a	9523.1.13	3-5	6	4.3±0.8 a-c
9521.1.1	-	-	-	9523.1.14	2-3	5	2.6±0.5 a-f
Mean			3.5±1.0	Mean			3.4±0.6

Table A.4. cont.

Plants	Range	N	Means
9524.1.1	1-4	3	2.3±1.5 b-f
9524.1.2	2-3	3	2.7±0.6 a-f
9524.1.4	3-3	3	3.0±0.0 a-f
9524.2.1	3-4	3	3.3±0.6 a-f
9524.2.2	-	-	-
9524.2.4	-	-	-
9524.2.5	3-4	3	3.3±0.6 a-f
9524.2.6	2-3	3	2.7±0.6 a-f
9524.2.7	1-3	3	1.7±1.2 d-f
9524.2.8	-	-	-
9524.2.9	2-3	3	2.7±0.6 a-f
9524.2.10	2-5	5	3.2±1.1 a-f
9524.2.11	-	-	-
9524.2.12	-	-	-
9524.2.13	4-5	3	4.7±0.6 ab
Mean			3.0±0.8
DFG50-7 TO	1-2	2	1.5±0.7 ef
Carrizo	2-3	3	2.3±0.6 b-f
Cleopatra	1-1	3	1.0±0.0 f

<sup>z</sup> Disease scoring: 1: no damaged roots,  
5: no healthy roots.

<sup>y</sup> Means associated with standard deviation  
with the same letter are not significantly  
different using Duncan's Multiple Range  
test,  $P \leq 0.05$ .

## Analysis of Variance

Source	DF	SS	MS	F Value	Pr > F
Model	69	171.757246	2.489235	1.75	0.0021
Error	160	227.116667	1.419479		
Corrected Total	229	398.873913			
R-Square		C.V.	Root MSE	Mean	
0.430605		36.68358	1.19142	3.24783	

Table A.5. *Phytophthora palmivora* Pp101 root rot disease infection rating of 'Wainwright' pummelo (9522) and 'Siamese' (9523) pummelo x DFG50-7 TO hybrids in the growth room on May 27, 1998<sup>z,y</sup>.

Plants	Range	N	Means	Plants	Range	N	Means
9522.1.1	2-5	9	3.3±1.0 b-g	9523.1.2	3-5	9	4.0±0.9 a-c
9522.1.2	2-4	7	3.0±0.8 c-g	9523.1.3	2-5	10	3.6±1.1 a-e
9522.1.5	-	-	-	9523.1.5	2-5	10	3.8±1.0 a-d
9522.1.6	3-4	9	3.6±0.5 a-f	9523.1.6	-	-	-
9522.1.7	3-4	9	3.6±0.5 a-f	9523.1.7	3-5	10	3.9±1.0 a-d
9522.1.8	2-5	9	3.3±1.2 b-g	9523.1.8	3-5	9	3.8±0.7 a-d
9522.1.9	3-5	8	4.6±0.7 a	9523.1.10	2-4	5	3.0±0.7 c-g
9522.1.10	3-5	8	4.0±0.8 a-c	9523.1.11	3-4	8	3.4±0.5 b-g
9522.1.11	2-5	9	3.3±1.0 b-g	9523.1.12	3-4	5	3.2±0.4 b-g
9522.1.12	2-5	9	3.9±1.1 a-d	9523.1.13	2-5	10	3.0±0.9 c-g
9522.1.13	2-5	10	3.9±0.9 a-d	9523.1.14	2-4	9	2.9±0.6 c-g
9522.1.15	2-5	8	3.3±1.0 b-g	Mean			3.5±0.4
9522.1.17	2-5	10	3.9±1.0 a-d	DFG50-7 TO	1-4	9	2.3±0.9 g
9522.1.21	2-5	10	3.4±1.2 b-g	Carrizo	2-4	6	2.8±1.0 d-g
9522.1.22	2-5	8	3.8±1.2 a-d	Cleopatra	2-5	10	2.9±1.1 c-g
9522.1.23	3-5	6	3.7±0.8 a-d				
9522.1.24	1-3	6	2.5±0.8 fg				
9522.2.1	2-4	10	2.9±0.7 c-g				
9522.2.2	3-5	7	4.6±0.8 a				
9522.2.3	2-3	9	2.6±0.5 e-g				
9522.2.4	2-5	10	3.9±1.2 a-d				
9522.2.5	-	-	-				
9522.2.6	3-5	10	4.3±0.8 ab				
Mean			3.6±0.6				

<sup>z</sup> Disease scoring: 1: no damaged roots, 5: no healthy roots.

<sup>y</sup> Means associated with standard deviation with the same letter are not significantly different at P-level using Duncan's Multiple Range test,  $P \leq 0.05$ .

#### Analysis of Variance

Source	DF	SS	MS	F Value	Pr > F
Model	33	85.8494600	2.6014988	3.17	0.0001
Error	257	210.8309524	0.8203539		
Corrected Total	290	296.6804124			
R-Square		C.V.	Root MSE	Mean	
0.289367		25.99296	0.90573	3.48454	

Table A.6. *Phytophthora palmivora* Pp101 root rot disease infection rating of 'Wainwright' pummelo (9522), 'Siamese' pummelo (9523), LB#6-1 ('Clementine' x 'Hamlin') (9648), and #8730 (('Clementine' x 'Minneola') x 'Orlando') (9669) x DFG50-7 TO hybrids in the greenhouse on Aug 11, 1998<sup>z,y</sup>.

Plants	Range	N	Means	Plants	Range	N	Means
9522.1.1	2-5	10	3.6±1.0 a-h	9648.1.3	1-5	4	2.8±1.7 d-l
9522.1.2	2-5	14	3.3±0.9 a-h	9648.1.14	3-4	3	3.3±0.6 a-j
9522.1.5	-	-	-	9648.1.15	3-5	3	4.0±1.0 a-d
9522.1.6	2-3	3	2.3±0.6 f-l	9648.1.19	1-5	6	3.0±1.3 b-k
9522.1.7	2-5	9	4.2±1.0 a-c	9648.1.20	3-4	3	3.7±0.6 a-g
9522.1.8	2-4	9	2.9±0.9 b-k	9648.1.26	3-5	4	4.3±1.0 ab
9522.1.9	3-5	10	3.8±0.9 b-k	9648.2.6	2-3	4	2.8±0.5 d-l
9522.1.10	2-5	8	3.6±1.4 a-g	9648.2.8	2-5	5	3.6±1.1 a-h
9522.1.11	2-2	5	2.0±0.0 j-l	9648.2.11	2-3	5	2.8±0.4 d-k
9522.1.12	2-4	10	3.3±0.7 a-j	9648.2.14	2-4	6	3.2±0.8 b-k
9522.1.13	3-4	11	3.5±0.5 a-i	9648.3.8	2-4	6	2.8±0.8 c-l
9522.1.15	2-5	9	3.7±1.0 a-g	9648.3.25	2-4	3	2.7±1.2 d-l
9522.1.17	3-5	9	3.3±0.7 a-j	9648.3.28	2-4	4	3.0±0.8 b-k
9522.1.21	1-5	13	2.8±1.1 b-l	9648.3.31	3-3	3	3.0±0.0 b-k
9522.1.22	2-3	12	2.4±0.5 e-l	9648.3.35	2-5	3	3.0±1.7 b-k
9522.1.23	3-5	9	3.8±1.0 a-c	9648.3.38	2-5	3	3.3±1.5 a-j
9522.1.24	-	-	-	9648.4.8	-	-	-
9522.2.1	2-5	12	3.4±0.9 a-i	9648.4.10	2-3	3	2.3±0.6 f-l
9522.2.2	2-5	12	3.8±1.1 a-f	9648.4.16	2-5	5	3.6±1.1 a-h
9522.2.3	1-4	9	2.6±1.1 e-l	9648.4.20	1-2	4	1.5±0.6 l
9522.2.4	4-5	6	4.7±0.5 a	9648.4.21	2-4	5	2.6±0.9 d-l
9522.2.5	-	-	-	9648.4.22	2-3	5	2.2±0.4 h-l
9522.2.6	2-5	12	3.8±1.1 a-f	9648.4.25	2-3	3	2.7±0.6 d-l
Mean			3.3±0.7	9648.5.5	2-3	4	2.3±0.5 h-l
9523.1.2	3-5	11	3.5±0.7 a-i	9648.5.7	3-5	5	3.6±0.9 a-h
9523.1.3	2-5	12	3.3±1.1 a-j	9648.5.12	2-3	3	2.7±0.6 d-l
9523.1.5	2-4	11	2.8±0.8 c-l	9648.5.13	1-3	4	2.3±1.0 g-l
9523.1.6	-	-	-	9648.5.18	3-4	4	3.5±0.6 a-i
9523.1.7	1-5	11	2.9±1.5 b-k	9648.5.20	2-4	11	3.1±0.5 b-k
9523.1.8	1-3	12	1.8±0.7 kl	9648.5.22	3-3	3	3.0±0.0 b-k
9523.1.10	-	-	-	9648.5.24	2-3	3	2.7±0.6 d-l
9523.1.11	2-5	17	3.0±1.1 b-k	9648.6.10	2-5	8	3.3±1.0 b-j
9523.1.12	-	-	-	Mean			3.0±0.6
9523.1.13	3-5	3	4.0±1.0 a-d				
9523.1.14	-	-	-				
Mean			3.0±0.7				

Table A.6. cont.

Plants	Range N		Means
9669.1.2	2-3	5	2.4±0.5 e-l
9669.2.8	3-4	3	3.3±0.6 a-j
9669.3.2	3-4	5	3.2±0.4 b-k
9669.5.2	3-4	4	3.3±0.5 b-k
9669.8.2	2-3	9	2.8±0.4 d-l
9669.10.7	3-4	4	3.8±0.5 a-f
9669.11.8	3-4	3	3.3±0.6 a-j
Mean			3.2±0.4
DFG50-7 TO	1-3	13	2.2±0.7 i-l
Carrizo	2-3	11	2.5±0.5 e-l
Cleopatra	1-3	11	2.0±0.6 j-l

<sup>z</sup> Disease scoring: 1: no damaged roots,

5: no healthy roots.

<sup>y</sup> Means associated with standard deviation with the same letter are not significantly different using Duncan's Multiple Range test,  $P \leq 0.05$ .

## Analysis of Variance

Source	DF	SS	MS	F Value	Pr > F
Model	67	175.996643	2.626816	3.30	0.0001
Error	404	321.780900	0.796487		
Corrected Total	471	497.777542			
R-Square		C.V.	Root MSE	Mean	
0.353565		28.95133	0.89246	3.08263	

Table A.7. *Phytophthora palmivora* Pp101 root rot disease infection rating of LB#6-1 ('Clementine' x 'Hamlin') (9648) and #8730 (('Clementine' x 'Minneola') x 'Orlando') (9669) x DFG50-7 TO hybrids in the greenhouse on Oct 23, 1998<sup>z,y</sup>.

Plants	Range	N	Means	Plants	Range	N	Means
9648.1.3	4-5	4	4.5±0.6 a-c	9669.1.2	4-4	5	4.0±0.0 a-f
9648.1.14	4-5	3	4.7±0.6 a-c	9669.2.8	4-5	4	4.5±0.6 a-c
9648.1.15	4-5	3	4.3±0.6 a-e	9669.3.2	3-5	5	4.0±1.0 a-f
9648.1.19	4-5	6	4.7±0.5 a-c	9669.5.2	3-4	4	3.3±0.5 d-f
9648.1.20	-	-	-	9669.8.2	4-5	9	4.4±0.5 a-d
9648.1.26	4-5	5	4.4±0.5 a-d	9669.10.7	4-5	4	4.8±0.5 a-c
9648.2.6	4-5	5	4.4±0.5 a-d	9669.11.8	3-4	3	3.7±0.6 b-f
9648.2.8	4-5	5	4.5±0.6 a-c	Mean			4.1±0.5
9648.2.11	5-5	5	5.0±0.0 a	DFG50-7 TO	3-5	5	3.8±0.8 a-f
9648.2.14	2-5	6	3.2±1.1 e-g	Carrizo	3-4	5	3.6±0.5 c-f
9648.3.8	3-5	6	4.5±0.8 a-c	Cleopatra	1-3	5	2.2±0.8 g
9648.3.25	1-4	3	3.0±1.7 fg	<sup>z</sup> Disease scoring: 1: no damaged roots, 5: no healthy roots.			
9648.3.28	5-5	4	5.0±0.0 a				
9648.3.31	5-5	3	5.0±0.0 a	<sup>y</sup> Means associated with standard deviation with the same letter are not significantly different using Duncan's Multiple Range test, P ≤ 0.05.			
9648.3.35	-	-	-				
9648.3.38	4-4	3	4.0±0.0 a-f				
9648.4.8	4-5	3	4.3±0.6 a-d				
9648.4.10	5-5	3	5.0±0.0 a				
9648.4.16	3-5	5	4.4±0.9 a-d				
9648.4.20	3-5	4	4.0±0.8 a-f				
9648.4.21	5-5	5	5.0±0.0 a				
9648.4.22	4-5	5	4.4±0.5 a-d				
9648.4.25	4-5	3	4.3±0.6 a-e				
9648.5.5	1-5	3	3.7±2.3 b-f				
9648.5.7	4-5	5	4.4±0.5 a-d				
9648.5.12	4-4	3	4.0±0.0 a-f				
9648.5.13	4-5	4	4.8±0.5 a-c				
9648.5.18	2-5	3	3.7±1.5 b-f				
9648.5.20	4-5	12	4.8±0.4 ab				
9648.5.22	4-5	3	4.3±0.6 a-e				
9648.5.24	5-5	3	5.0±0.0 a				
9648.6.10	4-5	8	4.5±0.5 a-c				
Mean			4.4±0.5				

#### Analysis of Variance

Source	DF	SS	MS	F Value	Pr > F
Model	39	65.6444444	1.6831909	3.52	0.0001
Error	140	66.9055556	0.4778968		
Corrected Total	179	132.5500000			
R-Square		C.V.	Root MSE	Mean	
0.495243		16.13932	0.69130	4.28333	

Table A.8. *Phytophthora nicotianae* Pn117 root rot disease infection rating of 'Wainwright' pummelo (9522), 'Siamese' pummelo (9523), LB#6-1 ('Clementine' x 'Hamlin') (9648), and #8730 (('Clementine' x 'Minneola') x 'Orlando') (9669) x DFG50-7 TO hybrids in the greenhouse on Oct 9, 1998<sup>z,y</sup>.

Plants	Range	N	Means	Plants	Range	N	Means
9522.1.1	1-2	11	1.2±0.4 fg	9648.1.3	1-1	3	1.0±0.0 g
9522.1.2	-	-	-	9648.1.14	2-3	3	2.3±0.6 c-f
9522.1.5	1-1	17	1.0±0.0 g	9648.1.15	-	-	-
9522.1.6	-	-	-	9648.1.16	1-1	3	1.0±0.0 g
9522.1.7	1-1	21	1.0±0.0 g	9648.1.19	-	-	-
9522.1.8	1-1	4	1.0±0.0 g	9648.1.20	1-2	3	1.3±0.6 fg
9522.1.9	4-5	6	4.7±0.5 a	9648.1.26	1-1	3	1.0±0.0 g
9522.1.10	1-2	5	1.2±0.4 fg	9648.2.6	-	-	-
9522.1.11	1-2	11	1.3±0.5 fg	9648.2.8	-	-	-
9522.1.12	1-3	10	1.8±0.8 e-g	9648.2.11	-	-	-
9522.1.13	1-1	9	1.0±0.0 g	9648.2.14	1-3	3	2.0±1.0 d-g
9522.1.15	1-3	8	1.4±0.7 fg	9648.2.16	1-3	3	1.7±1.2 e-g
9522.1.17	1-2	15	1.1±0.4 fg	9648.2.19	1-1	3	1.0±0.0 g
9522.1.21	2-5	16	3.6±1.1 b	9648.2.21	2-3	3	2.3±0.6 c-f
9522.1.22	1-1	13	1.0±0.0 g	9648.2.24	2-3	3	2.7±0.6 b-e
9522.1.23	-	-	-	9648.3.1	1-2	3	1.3±0.6 fg
9522.1.24	1-3	7	2.0±0.8 d-g	9648.3.8	2-5	3	3.0±1.7 b-d
9522.2.1	1-2	3	1.3±0.6 fg	9648.3.9	1-2	3	1.7±0.6 e-g
9522.2.2	1-1	10	1.0±0.0 g	9648.3.22	1-2	3	1.3±0.6 fg
9522.2.3	-	-	-	9648.3.24	1-1	3	1.0±0.0 g
9522.2.4	1-1	4	1.0±0.0 g	9648.3.25	-	-	-
9522.2.5	1-3	5	1.6±0.9 eg	9648.3.28	1-2	3	1.3±0.6 fg
9522.2.6	1-2	10	1.5±0.5 e-g	9648.3.31	-	-	-
Mean			1.6±1.0	9648.3.35	1-1	3	1.0±0.0 g
9523.1.2	2-5	9	3.3±1.2 bc	9648.3.38	-	-	-
9523.1.3	1-3	5	1.6±0.9 e-g	9648.4.4	1-2	3	1.3±0.6 fg
9523.1.5	-	-	-	9648.4.5	1-1	3	1.0±0.0 g
9523.1.6	1-3	21	1.8±0.7 e-g	9648.4.6	1-1	3	1.0±0.0 g
9523.1.7	1-1	4	1.0±0.0 g	9648.4.8	1-1	3	1.0±0.0 g
9523.1.8	1-1	5	1.0±0.0 g	9648.4.10	1-5	3	2.3±2.3 c-f
9523.1.10	1-1	9	1.0±0.0 g	9648.4.15	1-2	3	1.3±0.6 fg
9523.1.11	-	-	-	9648.4.16	-	-	-
9523.1.12	1-2	7	1.1±0.4 fg	9648.4.20	1-1	3	1.0±0.0 g
9523.1.13	-	-	-	9648.4.21	2-4	3	3.3±1.2 bc
9523.1.14	1-3	11	1.5±0.7 eg	9648.4.22	-	-	-
Mean			1.5±0.8	9648.4.25	1-3	3	2.0±1.0 d-g

Table A.8. cont.

Plants	Range	N	Means
9648.5.3	1-2	3	1.7±0.6 e-g
9648.5.5	-	-	-
9648.5.7	2-2	3	2.0±0.0 d-g
9648.5.9	2-3	3	2.3±0.6 c-f
9648.5.10	2-2	3	2.0±0.0 d-g
9648.5.12	-	-	-
9648.5.13	-	-	-
9648.5.18	-	-	-
9648.5.20	-	-	-
9648.5.22	-	-	-
9648.5.24	2-4	3	3.0±1.0 b-d
9648.6.10	-	-	-
Mean			1.7±0.7
9669.1.1	2-3	3	2.3±0.6 c-f
9669.1.2	-	-	-
9669.1.3	1-3	3	2.0±1.0 d-g
9669.1.9	1-2	3	1.3±0.6 fg
9669.2.1	1-2	3	1.7±0.6 e-g
9669.2.5	1-2	3	1.7±0.6 e-g
9669.2.6	1-2	3	1.3±0.6 fg
9669.2.8	1-2	3	1.7±0.6 e-g
9669.3.2	1-2	3	1.3±0.6 fg
9669.4.2	1-2	3	1.3±0.6 fg
9669.4.3	1-1	3	1.0±0.0 g

Plants	Range	N	Means
9669.4.4	1-4	3	2.3±1.5 c-f
9669.5.2	2-2	3	2.0±0.0 d-g
9669.6.4	1-2	3	1.3±0.6 fg
9669.6.5	1-2	3	1.3±0.6 fg
9669.6.8	1-2	3	1.3±0.6 fg
9669.7.1	1-2	3	1.7±0.6 e-g
9669.7.2	1-1	3	1.0±0.0 g
9669.8.2	1-2	3	1.3±0.6 fg
9669.9.2	1-3	3	2.7±1.2 b-e
9669.9.4	1-2	3	1.3±0.6 fg
9669.9.5	2-3	3	2.3±0.6 c-f
9669.9.6	1-2	3	1.3±0.6 fg
9669.10.2	1-2	3	1.3±0.6 fg
9669.10.6	1-2	3	1.3±0.6 fg
9669.10.7	2-3	3	2.3±0.6 c-f
9669.11.1	1-2	3	1.3±0.6 fg
9669.11.8	1-2	3	1.3±0.6 fg
Mean			1.6±0.5
DFG50-7 TO	1-1	14	1.0±0.0 g
Carrizo	2-5	13	3.4±1.1 bc
Cleopatra	2-5	18	3.8±1.1 ab

<sup>2</sup> Disease scoring: 1: no damaged roots, 5: no healthy roots.

<sup>3</sup> Means associated with standard deviation with the same letter are not significantly different using Duncan's Multiple Range test,  $P \leq 0.05$ .

Analysis of Variance

Source	DF	SS	MS	F Value	Pr > F
Model	87	377.623685	4.340502	9.71	0.0001
Error	387	173.007893	0.447049		
Corrected Total	474	550.631579			
R-Square		C.V.	Root MSE	Mean	
0.685801		38.96848	0.66862	1.71579	

Table A.9. Examples of some hybrids presenting different disease scores of *Phytophthora palmivora* Pp101 and *P. nicotianae* Pn117 inoculation experiments in citrus and trifoliate orange hybrids.

Hybrids	Oct 16, 1997	Nov 4, 1997	Jan 8, 1998	Mar 17, 1998	May 27, 1998	Aug 11, 1998	Oct 23, 1998	Oct 9, 1998
Pp101			Pp101	Pp101	Pp101	Pp101	Pp101	Pn117
10 prop/cm <sup>3</sup>	10 prop/cm <sup>3</sup>	10 prop/cm <sup>3</sup>	15 prop/cm <sup>3</sup>	20 prop/cm <sup>3</sup>	15 prop/cm <sup>3</sup>	5 prop/cm <sup>3</sup>	30 prop/cm <sup>3</sup>	45 prop/cm <sup>3</sup>
P<0.0205	P<0.0001	P<0.0001	P<0.0350	P<0.0001	P<0.0013	P<0.0001	P<0.0001	P<0.0001
9518.13.4	-	1.3±0.6 b-d	3.3±0.6 a-e	3.3±1.5 a-f	-	-	-	-
9518.7.2	-	2.0±1.6 a-d	4.1±0.6 a-d	4.3±1.2 a-c	-	-	-	-
9518.4.2	-	3.0±1.9 a-d	4.4±0.5 a	4.7±0.6 ab	-	-	-	-
9519.2.5	-	1.7±0.9 a-d	3.8±0.9 a-e	2.3±1.2 b-f	-	-	-	-
9519.2.1	-	1.8±0.6 b-d	2.4±1.1 e	3.3±1.5 a-f	-	-	-	-
9519.4.2	-	3.7±2.1 a	3.2±1.3 a-e	2.3±1.2 b-f	-	-	-	-
9522.1.22	-	1.0±0.0 g	-	4.3±1.2 a-c	3.8±1.2 a-d	2.4±0.5 e-l	-	1.0±0.0 g
9522.1.9	-	2.0±0.7 a-g	-	4.3±0.1.2 a-c	4.6±0.7 a	3.8±0.9 b-k	-	4.7±0.5 a
9522.2.4	-	3.0±1.4 a	-	3.4±1.1 a-f	3.9±1.2 a-d	4.7±0.5 a	-	1.0±0.0 g
9523.1.8	-	1.2±0.4 d-g	-	3.0±1.0 a-f	3.8±0.7 a-d	1.8±0.8 kl	-	1.0±0.0 g
9523.1.2	-	1.3±0.6 d-g	-	3.3±0.6 a-f	4.0±0.9 a-c	3.5±0.7 a-i	-	3.3±1.2 bc
9523.1.7	-	1.7±1.0 b-g	-	3.3±1.5 a-f	3.9±1.0 a-d	2.9±1.5 b-k	-	1.0±0.0 g
9524.2.9	-	1.0±0.0 d	3.7±0.5 a-e	2.7±0.6 a-f	-	-	-	-
9524.1.1	-	2.2±1.2 a-d	4.0±1.1 a-d	2.3±1.5 b-f	-	-	-	-
9524.2.5	-	2.5±1.7 a-d	3.8±0.5 a-e	3.3±0.6 a-f	-	-	-	-
9648.5.24	-	-	-	-	-	2.7±0.6 d-l	5.0±0.0 a	3.0±1.0 b-d
9648.3.28	-	-	-	-	-	3.0±0.8 b-k	5.0±0.0 a	1.3±0.6 fg
9648.1.26	-	-	-	-	-	4.3±0.1 ab	4.4±0.5 a-d	1.0±0.0 g
9669.1.2	-	-	-	-	-	2.4±0.5 e-l	4.0±0.0 a-f	2.3±0.6 c-f
9669.3.2	-	-	-	-	-	3.2±0.4 b-k	4.0±1.0 a-f	1.3±0.6 fg
9669.10.7	-	-	-	-	-	3.8±0.5 a-f	4.8±0.5 a-c	2.3±0.6 c-f

Table A.10. Regression analysis and correlation of experiment dates for *Phytophthora palmivora* Pp101, *P. nicotianae* Pn117, and *P. nicotianae* Hall 3 disease infection ratings of citrus and trifoliate orange hybrids.

Dates	$Y=\beta_0+\beta_1X$	r	r <sup>2</sup>
9522, 9523			
Oct 16, 97 vs Mar 17, 98	0.44-0.22X	-0.13	0.0167
Oct 16, 97 vs May 27, 98	3.74-0.11X	-0.11	0.0119
Oct 16, 97 vs Aug 11, 98	2.30+0.47X	0.34	0.1179
Oct 16, 97 vs Oct 9, 98	2.21-0.26X	-0.13	0.0167
Mar 17, 98 vs May 27, 98	2.50+0.30X	0.47	0.2222
Mar 17, 98 vs Aug 11, 98	2.29+0.27X	0.34	0.1137
Mar 17, 98 vs Oct 9, 98	1.57+0.05X	0.04	0.0014
May 27, 98 vs Aug 11, 98	1.23+0.54X	0.42	0.1776
May 27, 98 vs Oct 9, 98	1.43+0.08X	0.04	0.0018
Aug 11, 98 vs Oct 9, 98	2.35-0.19X	-0.13	0.0165
Mean Pp vs Oct 9, 98	1.89-0.08X	-0.04	0.0018
9518, 9519-21, 9524			
Nov 4, 97 vs Jan 8, 98	3.69-0.07X	-0.07	0.0053
Nov 4, 97 vs Mar 17, 98	3.14+0.05X	0.03	0.0008
Jan 8, 98 vs Mar 17, 98	1.11+0.60X	0.32	0.1045
9648, 9669			
Aug 11, 98 vs Oct 9, 98	3.05-0.38X	-0.28	0.0757
Aug 11, 98 vs Oct 23, 98	3.64+0.20X	0.20	0.0391
Oct 9, 98 vs Oct 23, 98	4.46-0.12X	-0.15	0.0240
Sunki x FD			
Sep 30, 97 vs Jan 25, 99	1.67+0.38X	0.48	0.2312
Sep 30, 97 vs Apr 13, 99	2.52+0.13X	0.16	0.0256
Jan 25, 99 vs Apr 13, 99	2.14+0.27X	0.26	0.0676
Pearl x FD			
Sep 30, 97 vs Jan 25, 99	2.57+0.42X	0.39	0.1494
Sep 30, 97 vs Apr 13, 99	2.78+0.36X	0.60	0.3642
Jan 25, 99 vs Apr 13, 99	2.41+0.34X	0.63	0.3966

Table A.11. Orthogonal contrasts between the means of citrus and trifoliate orange crosses, and control plants.

Plants	[ <i>l<sub>i</sub></i> ] <sup>2</sup>			V( <i>l<sub>i</sub></i> )	<i>t<sub>i</sub></i>				
Oct 16, 1997, Pp101, df=64, <i>t</i> <sub>0.005</sub> =2.657									
(9522+9523) vs. DFG50-7 TO	0.7			0.08	2.53				
(9522+9523) vs. Carrizo	0.1			0.07	0.38				
(9522+9523) vs. Cleopatra	0.1			0.07	0.39				
Nov 4, 1997, Pp101, df=62, <i>t</i> <sub>0.005</sub> =2.656									
(9518+9519+9524) vs. DFG50-7 TO	1.1			0.04	5.62*				
(9518+9519+9524) vs. Carrizo	0.8			0.06	3.29*				
(9518+9519+9524) vs. Cleopatra	0.8			0.04	4.09*				
Jan 8, 1998, Pp101, df=59, <i>t</i> <sub>0.005</sub> =2.662									
(9518+9519+9524) vs. DFG50-7 TO	0.2			0.07	0.73				
(9518+9519+9524) vs. Carrizo	0.2			0.11	0.59				
(9518+9519+9524) vs. Cleopatra	0.9			0.07	3.23*				
Mar 17, 1998, Pp101, df=67, <i>t</i> <sub>0.005</sub> =2.655									
(9518+9519+9522+9523+9524) vs. DFG50-7 TO	1.8			0.33	3.11*				
(9518+9519+9522+9523+9524) vs. Carrizo	1.0			0.23	4.42*				
(9518+9519+9522+9523+9524) vs. Cleopatra	2.3			0.23	4.83*				
May 27, 1998, Pp101, df=51, <i>t</i> <sub>0.005</sub> =2.680									
(9522+9523) vs. DFG50-7 TO	1.3			0.09	4.41*				
(9522+9523) vs. Carrizo	0.8			0.12	2.31				
(9522+9523) vs. Cleopatra	0.7			0.08	4.47*				
Aug 11, 1998, Pp101, df=93, <i>t</i> <sub>0.005</sub> =2.636									
(9522+9523+9648+9669) vs. DFG50-7 TO	0.9			0.04	4.65*				
(9522+9523+9648+9669) vs. Carrizo	0.6			0.04	2.91*				
(9522+9523+9648+9669) vs. Cleopatra	1.1			0.04	5.33*				
Oct 23, 1998, Pp101, df=47, <i>t</i> <sub>0.005</sub> =2.689									
(9648+9669) vs. DFG50-7 TO	0.5			0.08	1.74				
(9648+9669) vs. Carrizo	0.7			0.08	2.43				
(9648+9669) vs. Cleopatra	2.1			0.08	7.30*				
Oct 9, 1998, Pn117, df=123, <i>t</i> <sub>0.005</sub> =2.616									
(9522+9523+9648+9669) vs. DFG50-7 TO	0.6			0.05	2.56				
(9522+9523+9648+9669) vs. Carrizo	1.8			0.06	7.46*				
(9522+9523+9648+9669) vs. Cleopatra	2.2			0.04	10.39*				
Sep 30, 1997, Hall 3 df=88, <i>t</i> <sub>0.005</sub> =2.647									
Jan 25, 1999, Pp101 df=21, <i>t</i> <sub>0.005</sub> =2.831									
Apr 13, 1999, Pp101 df=21, <i>t</i> <sub>0.005</sub> =2.831									
	[ <i>l<sub>i</sub></i> ]	V( <i>l<sub>i</sub></i> )	<i>t<sub>i</sub></i>	[ <i>l<sub>i</sub></i> ]	V( <i>l<sub>i</sub></i> )	<i>t<sub>i</sub></i>	[ <i>l<sub>i</sub></i> ]	V( <i>l<sub>i</sub></i> )	<i>t<sub>i</sub></i>
S x FD vs. Swingle	0.82	0.01	7.87*	1.20	0.06	5.00*	1.00	0.03	6.14*
P x FD vs. Swingle	0.65	0.01	6.40*	2.10	0.06	8.49*	1.78	0.03	10.60*

<sup>2</sup> [ $l_i$ ]: Positive difference between the means, V( $l_i$ ): Variance,  $t_i$ : Calculated t-value. Mean comparisons were made at  $\alpha_{0.01}$  level.  $t_{\alpha/2}=t_{0.005}$  value was given next to each scoring date according to its corresponding degree of freedom (df).

\* Differences between means are significant at  $t_{\alpha/2}=t_{0.01/2}=t_{0.005}$  level.

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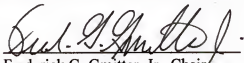
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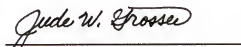
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Zeynel Dalkılıç was born on June 25, 1969 in Eskişehir, Turkey. He completed his undergraduate degree at Uludağ University, College of Agriculture, Department of Horticulture in Bursa, Turkey in 1990. During his three-year master of science study at the same university, he served as a research assistant. In 1993, Zeynel was awarded a scholarship from the Turkish High Education Counsel (YÖK) for his Ph.D. study abroad. He took six-month English course at American Language Academy, Tampa, FL. Then, he matriculated to the University of Florida, Gainesville in June 1994. After finishing his course work, he moved to UF-IFAS-Citrus Research and Education Center, Lake Alfred, where he conducted his research. After completing his Ph.D., he will take a position at Adnan Menderes University in Aydın, Turkey.

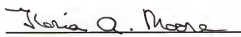
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Frederick G. Gmitter, Jr., Chair  
Professor of Horticultural  
Sciences

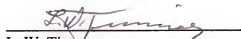
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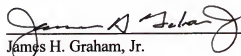
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1999



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